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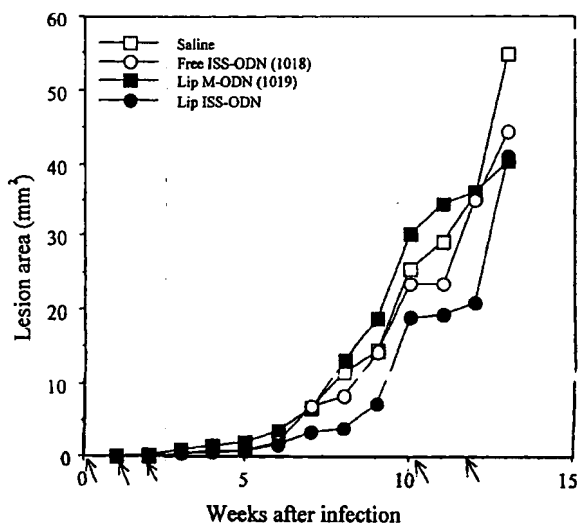
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(54) Title: A METHOD FOR PREPARATION OF VESICLES LOADED WITH IMMUNOSTIMULATORY OLIGODEOXYNUCLEOTIDES AND DIFFERENT USES THEREOF



(57) Abstract: The present invention is based on the finding of a simple and fast method for an effective entrapment of active ISS-ODN in liposomes, with more than 60% loading). The method is based on drying a suspension of amphipathic material and then hydrating it with an aqueous solution containing the ISS-ODN thereby entrapping it in liposomes formed from the lipid. Thus, the present invention concerns an efficient method for loading, and retaining, ISS-ODN in liposomal vesicles. Animals treated with liposomal ISS-ODN of the invention are shown to demonstrate a slower tempo of disease development as compared to free ISS-ODN or to liposomes loaded with mutant ISS-ODN (lip M-ODN).

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**A METHOD FOR PREPARATION OF VESICLES LOADED WITH
IMMUNOSTIMULATORY OLIGODEOXYNUCLEOTIDES AND DIFFERENT
USES THEREOF**

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FIELD OF THE INVENTION

This invention generally relates to liposomal formulations and in particular to a method for the preparation of liposome vesicles loaded with immunostimulatory oligodeoxynucleotides (ISS-ODN) and to the different uses of its products.

10 **PRIOR ART**

The following is a list of prior art which is considered to be pertinent for describing the state of the art in the field of the invention.

- (1) Lichtenberg D., and Barenholz Y. in *Methods of Biochemical Analysis* (Glick D., Ed.) Wiley NY pp. 337-462, 1988;
- 15 (2) Barenholz Y., and Crommelin D.J.A., in *Encyclopeida of Pharmaceutical Technology* (Swabrick J and Boylan J.C. Eds.) Vol. 9, Marcel Dekker NY pp. 1-39 (1994);
- (3) US Patent No. 6,156,337;
- (4) US Patent No. 6,066,331;
- 20 (5) C. Kirby and G. Gregoriadis *Bio/Technology*, November 1984, pages 979-984;
- (6) Van Uden J., and Raz, E. in *Springer Semin. Immunopathol.* **22**: 1-9 (2000);
- (7) McCluskie, M.J., *et al. Vaccine*, **19**:2657-2660 (2001);
- (8) Horner, A.A., *et al. Immunol Rev.* **179**,102-118 (2001);
- (9) Klinman, D.M., *et al. Springer Semin. Immunopathol* **22**:173-183 (2000);
- 25 (10) Wagner, H., *et al. Springer Semin. Immunopathol.* **22**:167-171 (2000);
- (11) Diminsky, *et al., Vaccine*, **15**:637-647 (1997);

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- (12) Alving, CR. (1997) in *New generation vaccines*, 2nd ed. (Levine, M.M., Woodrow, G.C., Kaper, J.B., and Cobon, G.S., eds.), Marcel Dekker, New York, pp. 207-213;
- (13) Kedar, E. and Barenholz, Y. (1998) in *The biotherapy of cancers: from immunotherapy to gene therapy* (Chouaib S, ed.), INSERM, Paris, pp. 333-362.

BACKGROUND OF THE INVENTION

Several attempts have been made to use lipid vesicles formed by natural or synthetic phospholipids as vehicles for the administration of effective substances. Proposed clinical uses have included vaccine adjuvanticity, gene transfer and diagnostic imaging, but the major effort has been in the development of liposomes as targetable drug carriers in the treatment of malignancy.

Amphotericin B, an effective but toxic antifungal, was the first liposomally formulated agent to be licensed for parenteral use.

Antitumor agents like adriamycin (doxorubicin) have also been incorporated into liposomes, as well as vaccines, adjuvants and biological response modifiers like cytokines and others.

Liposomes are also utilized as vehicles in the field of gene transfer [Kastel P.L, and Greenstein R.J., *Biotechnol. Annu. Rev.* 5:197-220 (2000)]. In another application, liposomes were used for the delivery of therapeutic proteins. N. Sakuragawa *et al.* [Thrombosis Research 38:681-685, (1985); Clinical Hematology 29(5):655-661 (1988)] report that liposomes containing factor VIII have been prepared for oral administration to patients suffering from von Willebrand's disease.

The encapsulation of factor VIII was carried out by dissolving the protein factor VIII concentrates in an aprotinin containing solution and transferred into lecithin coated flasks. After drying the flasks by rotation for 30 min under negative pressure liposomes were formed which entrapped factor VIII concentrates. The liposome suspension was centrifuged yielding 40% of factor VIII entrapped in liposomes.

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Another method for entrapment of drugs in liposomes is based on a procedure referred to as the dehydration-re-hydration procedure. This is described by C. Kirby and G. Gregoriadis [Bio/Technology, November 1984, pages 979-984]. In this preparation the entrapment was increased by using additional lipid and the use of cholesterol is
5 described as having positive influence of the drug entrapment.

Yet another method for loading vesicles with biological substances is described in US Patent Nos. 6,066,331 and 6,156,337. According to the method described therein, liposomes loaded with biological structures, biopolymers and/or oligomers, are obtained by co-drying a fraction of an amphipathic material in an organic solvent and a fraction of
10 the biological structure(s), biopolymers and/or oligomers, from an aqueous medium.

The present invention aims for the providence of a novel method for efficient encapsulation ($\geq 60\%$) of oligomers, particularly those being pharmaceutically active, into lipid membrane vesicles.

A group of oligomers of particular interest according to the present invention are
15 oligonucleotides and especially, the immunostimulatory oligodeoxynucleotides and analogs (ISS-ODN or CpG motifs). Typically, ISS-ODN are short synthetic oligodeoxynucleotides (6-30 bases) usually containing an active 6-mer sequence that has the general structure of two 5' purines, an unmethylated CpG dinucleotide, and two 3' pyrimidines (Pu-Pu-CpG-Pyr-Pyr).

20 Bacterial DNA and its synthetic ISS-ODN are known to be potent stimulators of both innate immunity and specific adaptive immune responses, including direct activation of monocytes/macrophages, dendritic cells, NK cells and B cells. Further, bacterial DNA and its synthetic ISS-ODN induce the production of pro-inflammatory cytokines (e.g., IL-6, IL-12, IFNs, $\text{TNF}\alpha$) and up-regulate the expression of MHC I,
25 MHC II and co-stimulatory molecules [Van Uden J., and Raz, E. in *Springer Semin. Immunopathol.* 22:1-9 (2000)].

In animal studies, ISS-ODNs exhibit strong Th1 and mucosal adjuvanticity to a wide range of antigens [McCluskie, M.J., *et al. Vaccine*, 19:2657-2660 (2001)] or

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allergens [Horner, A.A., *et al. Immunol Rev.* 179:102-118 (2001)]. Furthermore, pretreatment with ISS-ODN, even without concomitant administration of the relevant antigen, was shown to afford protection (for about 2 weeks) against subsequent infection with intracellular pathogens [Klinman, D.M., *et al. in Springer Semin. Immunopathol*
5 22:173-183 (2000)], indicating activation of innate immunity.

The immunostimulatory activity of ISS-ODNs requires cellular uptake by endocytosis following their binding to a receptor belonging to the Toll-like receptor family, TLR9. Endosomal acidification and digestion of the ODN followed by interaction with specific protein kinases results in rapid generation of reactive oxygen
10 intermediates, leading to activation of MAPK and NF- κ B pathways and subsequent cytokine production (Chu, W., *et al. Cell* 103:909-918 (2000)).

In mice, doses of 50-100 μ g per mouse of soluble ISS-ODN, and in many cases two or more administrations were required to achieve the desired immunomodulatory effects. This relatively high dose and repeated administration, in theory, may cause
15 adverse reactions resulting from the "cytokine storm" induced [Wagner, H., *et al. Springer Semin. Immunopathol.* 22:167-171 (2000)].

As liposomes can effectively entrap various drugs, which are slowly released over an extended period of time *in vivo*, and can rapidly and effectively be uptaken by macrophages and dendritic cells, it is suggested that liposomes can serve as an efficient
20 delivery system for ISS-ODN based vaccines [Alving, CR. (1997) in *New generation vaccines*, 2nd ed. (Levine, M.M., Woodrow, G.C., Kaper, J.B., and Cobon, G.S., eds.), Marcel Dekker, New York, pp. 207-213; and Kedar, E. and Barenholz, Y. (1998) in *The biotherapy of cancers: from immunotherapy to gene therapy* (Chouaib S, ed.), INSERM, Paris, pp. 333-362].

SUMMARY OF THE INVENTION

The present invention is based on the surprising finding that step-wise hydration of lipids, *a priori* freeze dried, with an aqueous solution containing ISS-ODN to be encapsulated in said liposomes, results in a very effective encapsulation of the ISS-ODN
5 as compared to hitherto known encapsulation methods.

Thus, according to a first of its aspects, the present invention provides a method for loading ISS-ODN in liposomes, the method comprising:

- (1) solubilizing at least one liposome-forming lipid in a solvent and drying the same to effect a dry liposome-forming lipid or a dry mixture of such lipids;
- 10 (2) providing an aqueous solution of ISS-ODN or of a mixture of ISS-ODNs;
- (3) hydrating said liposome-forming lipid or said liposome-forming lipids with said ISS-ODN to yield liposomes loaded with said ISS-ODN.

The term "*liposome*" as used includes all spheres or vesicles of amphipathic substance which may spontaneously or non-spontaneously vesiculate, for example
15 phospholipids, where at least one acyl group is replaced by a complex phosphoric acid ester.

The term "*loading*" means any kind of interaction of the oligomeric substances to be loaded with the liposomes, for example, an interaction such as encapsulation, adhesion, entrapment (to the inner or outer wall of the vesicle) or embedding in the
20 liposome membrane, with or without extrusion of the ISS-ODN.

Also as used herein, the term "*liposome-forming lipid*" denotes any physiologically acceptable amphipathic substance that contains groups with characteristically different properties, e.g. both hydrophilic and hydrophobic properties or a mixture of such molecules, and which upon dispersion thereof in an aqueous
25 medium, form vesicles. As will be further elaborated hereinafter, this term refers to a single amphipathic substance or to a mixture of such substances. The amphipathic substance includes, *inter alia*, phospholipids, sphingolipids, glycolipids, such as

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cerebrosides and gangliosides; PEGylated lipids and sterols, such as cholesterol and others.

The terms "*dry*" or "*drying*" refer to any manner of drying the liposome-forming lipids, which results in the formation of a dry lipid cake. According to one preferred
5 embodiment, drying is achieved by freeze drying, also referred to as lyophilizing. Alternatively, drying may be achieved by spray drying.

The term "*solubilizing*" which is used herein interchangeably with the term "*dissolving*" or "*dispersing*" may be achieved by a single use of the bulk aqueous medium with which said solubilization is achieved. However, this term preferably refers
10 to step-wise addition of two or more aliquots of the said medium solubilizing the solute.

The method of the invention will at times be referred to in the following description by the term "*post-encapsulation*" ("POST"), according to which dry lipids are hydrated with an aqueous solution containing the ISS-ODN. This is as opposed to Co-encapsulation technique. "*Co-encapsulation*" ("CO") is an encapsulation method,
15 which includes co-drying the liposome forming lipid(s) and the ISS-ODN (co-lyophilized) after which they are hydrated together with an aqueous medium.

One feature of the *post encapsulation* methodology disclosed herein is that it does not necessitate the freeze-drying of the ISS-ODN. As may be appreciated, there are numerous biological substances which are sensitive to lyophilization, which procedure
20 result in deactivation of the biological substance. In addition, by the method of the present invention ISS-ODN does not need to be exposed to any organic solvent or detergent that may be destructive to its activity.

The method of the present invention enables to obtain vesicles with substantially high loading rate of ISS-ODNs (more than 60%). This feature is unique since it improves
25 efficiency of treatment or prophylaxis with ISS-ODN loaded into the liposomes as well as it enables to reduce the dose and frequency/number of administrations required in order to achieve a desired therapeutic effect.

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Further, since by the present invention, the liposome-forming lipids and the ISS-ODN are kept separately, it enables combinatorial formulations, i.e. the physician may prescribe any combination of liposome-forming(s) substance and biological agent, and upon need, the pharmacist can easily formulate the selected combination.

5 Further, by the present invention, the freeze-dried lipids are provided with a long shelf-life at 4°C or at room temperature, preserving their entrapment capability for over two years (data not shown), and the hydration of the lipids with the ISS-ODN solution to form the liposomes is very simple and requires only several minutes. Therefore, the liposomal formulation can be readily prepared before treatment (bedside), ensuring high
10 pharmaceutical stability of the formulation and without leakage of the entrapped material from the liposome.

According to a second aspect, there is provided by the present invention a combination of two compositions including a first composition comprising dry liposome-forming lipids and a second composition comprising ISS-ODN, the combination is for
15 use in the preparation of a pharmaceutical formulation comprising liposomal ISS-ODN.

The combination of the invention may be obtained in the form of a package. Accordingly, the present invention also concerns a combination of at least one composition of dry liposome-forming lipid or a dry mixture of liposome-forming lipids; and at least one composition comprising ISS-ODN; further comprising instructions for
20 selection and use of the first and second compositions for the preparation of a therapeutic formulation, said instructions comprising hydrating said dry liposome-forming lipid(s) with an aqueous solution of said ISS-ODN to yield a pharmaceutical formulation comprising liposomes loaded with ISS-ODN; and further comprising instructions prescribing administration of said pharmaceutical formulation to a subject in need of said
25 formulation.

The present invention also concerns pharmaceutical formulations comprising as active ingredient a therapeutically effective amount of liposomal ISS-ODN; the liposomal ISS-ODN being prepared by the method of the invention as disclosed hereinabove and below.

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The pharmaceutically "*effective amount*", including also a prophylactically effective amount, for purposes herein, is determined by such considerations as are known in the art. The amount refers to that of ISS-ODN must be effective to achieve the desired therapeutic effect.

5 According to yet a further aspect of the invention there are provided therapeutic methods comprising administration to a subject in need an effective amount of liposomal ISS-ODN prepared according to the present invention, optionally in combination with other active agents, such as antigens. According to one embodiment, the therapeutic method comprises the prevention or treatment of a disease.

10 The terms "*prevention or treatment*" or "*treatment*" as used herein refer to administering of a therapeutic amount of liposomal ISS-ODN which is effective to ameliorate undesired symptoms associated with a disease, to prevent the manifestation of such symptoms before they occur, to slow down the progression of the disease, slow down the deterioration of symptoms, to enhance the onset of remission period, slow
15 down the irreversible damage caused in the progressive chronic stage of the disease, to delay the onset of said progressive stage, to lessen the severity or cure the disease, to improve survival rate or more rapid recovery, to prevent the disease from occurring, to prime the immune response against the disease to be treated, or a combination of two or more of the above. In addition, the term "*treatment*" in the context used herein refers to
20 prevention of a disease from occurring. The treatment (also preventative treatment) regimen and the specific formulations to be administered will depend on the type of disease to be treated and may be determined by various considerations known to those skilled in the art of medicine, e.g. the physicians.

Finally, the invention concerns a method for achieving a therapeutic effect, the
25 therapeutic effect comprising stimulating an immune response of an individual, the method comprising administration to said individual an amount of liposomal ISS-ODN effective to achieve said therapeutic effect, wherein said effect is to an extent greater than that obtained by administration to the individual free ISS-ODN, the liposomal ISS-IDN being prepared by the method of the invention.

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The amount administered to the individual, is a dosage of up to 2,000 mg of ISS-ODN loaded liposomal vesicles (measured by phospholipid), per kg body weight (wt).

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows the therapeutic effect of Leishmanial infection in mice by free
 5 ISS-ODN (ODN 1018, ○), liposomal M-ODN (ODN 1019, ■), or liposomal ISS-ODN, (●), Saline served as the control (□). Leishmania major promastigotes (250,000) are injected s.c. on day 0; i.m. injection of ODN (20 ug, free or liposomal) on day 1, 7, 14, 72 and 82 (⚡); n=10 Balb/c mice per group.

Fig. 2 shows the therapeutic effect of Leishmanial infection by a free
 10 anti-Leishmania drug, Amphotericin B (Fungizone, ○), liposomal ISS-ODN (ODN 1018, ■), or liposomal ISS-ODN in combination with Fungizone (●); Saline served as the control (□). Leishmania major promastigotes (250,000) were injected s.c. on day 0; ODN was injected i.m. (20 ug, free or liposomal) on day 7, 14, 21, and 28 (⚡); and Fungizone was injected s.c. (20 ug) on day 7, 9, 12, 15 and 18; n=10 Balb/c mice per
 15 group.

Fig. 3 shows the therapeutic effect of Leishmanial infection by free Amphotericin B derivative (AmB-AG, ●), Liposomal M-ODN (ODN 1019, ■), liposomal ISS-ODN (ODN 1018, ○), and liposomal ISS-ODN in combination with AmB-AG (△); Saline served as the control (□). Leishmania major promastigotes (250,000) were injected s.c.
 20 on day 0; ODN was injected i.m. (20 ug, free or liposomal) on day 1, 7, 14, and 21 (⚡); and AmB-AG was injected s.c. (20 ug) on day 1, 3, 5, and 7; n=10 Balb/c mice per group

DETAILED DESCRIPTION OF THE INVENTION

Liposomes can be classified according to various parameters. For example, when size and number of lamellae (structural parameters) are used, four major types of
 25 liposomes are identified: multilamellar vesicles (MLV), small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and oligolamellar vesicles (OLV).

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MLV form spontaneously upon hydration of dried phospholipids above their gel to liquid crystalline phase transition temperature (T_m). Their size and shape are heterogeneous and their exact structure is determined by their method of preparation [Barenholz, Y. and Crommelin, D.J.A., (1994) *ibid.*]. In general, MLV have an aqueous and lipid component separated by bilayers.

SUV are formed from MLV by sonication or by extrusion and are single bilayered (<100 nm). They are the smallest species with a high surveillance and high surface-to-volume ratio and hence have the lowest capture volume of aqueous space to weight of lipid.

The third type of liposome according to this classification includes large (> 100 nm) unilamellar vesicles (LUV) having one large aqueous compartment and a single lipid bilayer, while the fourth type of liposome includes oligolamellar vesicles (OLV), which are vesicles containing few lamellae (lipid bilayers).

Liposomes are formed from amphipathic compounds, which may spontaneously or non-spontaneously vesiculate. Such amphipathic compounds typically include triacylglycerols where at least one acyl group is replaced by a polar and/or charged moiety, e.g. phospholipids formed by a complex phosphoric acid ester. Other groups of liposome-forming lipids are the sphingolipids such as sphingomyelin (N-acyl sphingosine-phosphocholine). Examples of sphingomyelins (SPM) include (but are not limited to) egg-derived SPM, milk-derived SPM, N-palmitoyl-SPM, N-stearoyl-SPM, N-oleoyl-SPM (C18:1), N-nervacyl C (C24:1) SPM, N-lignoceryl SPM (C24:0), and their mixtures.

Any commonly known liposome-forming lipid may be suitable for use by the method of the present invention. The liposome-forming lipids can also be produced synthetically. The source of the lipid or its method of synthesis is not critical: any naturally occurring lipid, with and without modification, or a synthetic phosphatide can be used.

According to one embodiment, the lipids are phospholipids.

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Examples of specific phosphatides are L- α -(distearoyl) lecithin, L- α -(diapalmitoyl) lecithin, L- α -phosphatide acid, L- α -(dilauroyl)-phosphatidic acid, L- α -(dimyristoyl) phosphatidic acid, L- α -(dioleoyl)phosphatidic acid, DL- α -(dipalmitoyl) phosphatidic acid, L- α -(distearoyl) phosphatidic acid, and the various types of L- α -
5 phosphatidylcholines prepared from brain, liver, egg yolk, heart, soybean and the like, or synthetically, and salts thereof. Other suitable modifications include the controlled peroxidation of the fatty acyl residue cross-linkers in the phosphatidylcholines (PC) and the zwitterionic amphiphates which form micelles by themselves or when mixed with the PCs such as alkyl analogues of PC.

10 According to one embodiment, lecithines (also known as phosphatidylcholines (PC)) are used, which are mixtures of the diglycerides of stearic, palmitic, and oleic acids linked to the choline ester of phosphoric acid. The lecithines are found in all animals and plants such as eggs, soybeans, and animal tissues (brain, heart, and the like). These can be modified by partial or complete hydrogenation to produce partial or fully
15 hydrogenated phospholipids (i.e. hydrogenated soy phosphatidylcholine).

The phospholipids can vary in purity and can also be hydrogenated either fully or partially. Hydrogenation reduces the level of unwanted peroxidation, and modifies and controls the gel to liquid/crystalline phase main transition temperature (T_m) which effects packing and leakage.

20 The liposomes can be "tailored" to the requirements of any specific reservoir including various biological fluids, which maintain their stability without aggregation or chromatographic separation, and thereby remain well dispersed and suspended in the injected fluid. The fluidity *in situ* changes due to the composition, temperature, salinity, bivalent ions and presence of proteins. The liposomes can be used with or without any
25 other solvent or surfactant.

The liposomes may contain a combination of lipid components, as long as this combination does not induce instability, aggregation and/or chromatographic separation (demixing). This can be determined by routine experimentation, known to those in the art.

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A variety of methods for producing the different types of liposomes are known and available of such methods include, for example:

1. Hydrating a thin dried film of a phospholipid with an aqueous medium followed by mechanical shaking, ultrasonic irradiation and/or extrusion of the liposome thus formed through a suitable filter;
2. Dissolving a lipid in a suitable organic solvent, mixing with an aqueous medium followed by removal of the solvent;
3. By use of a gas above its critical point (i.e., freon and other gases such as CO₂ or mixtures of CO₂ and other gaseous hydrocarbons) or
4. Preparing lipid detergent mixed micelles followed by lowering the concentration of the detergent to a level below its critical concentration at which liposomes are formed [Lichtenberg D and Barenholz Y. (1988) *ibid.*].
5. Hydrating (dispersing) with aqueous medium dry liposomes loaded with an active agent (US 66,066,331, US 6,156,337).

One obstacle when using liposomes as a drug delivery tool, are the potential destructive/inactivating effect of the loading process on the biological material to be loaded into the liposome and the efficiency of loading of the biologically effective material, and the physical stability of the liposomal formulation with storage time. For water-soluble expensive drugs passively loaded into the intraliposomal aqueous phase, the best loading available to date is $\leq 60\%$. Non-efficient loading leaves a large amount of the drug un-encapsulated, and when the drugs are toxic and/or expensive this un-encapsulated drug is a major drawback. Therefore, an additional step of removal of the free drug is required, which adds unwanted handling and cost to the process of preparation of liposome formulation. A second drawback for methods of solvent or detergent removal is residual level of unwanted solvents or detergents.

The present invention provides a novel, fast and simple method for preparing liposomes efficiently loaded (i.e. at least 60% loading) with ISS-ODN.

Because of the fast clearance/degradation of "free" ISS-ODN after administration, effective encapsulation of the molecule, as achieved by the method of the present

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invention, may reduce the number of doses and frequency of administrations required in order to achieve a desired therapeutic effect.

Liposomal ISS-ODN as obtained by the present invention may be utilized, for example, as a vaccine adjuvant against pathogens and cancer; in therapeutic treatment or
5 prevention of diseases caused by certain infectious microorganisms; in the treatment or prevention of allergic diseases; or to boost innate immunity. According to one embodiment, the ISS-ODN is an endotoxin-free ISS-ODN with a phosphorothioate (PS) or phosphodiester (PO) backbone.

According to the invention, liposomal ISS-ODN are prepared by:

- 10 (a) solubilizing at least one liposome-forming lipid in a solvent and drying same to effect a dry liposome-forming lipid or a dry mixture of liposome-forming lipids;
- (b) providing an aqueous solution of ISS-ODN or of a mixture of ISS-ODNs; and
- 15 (c) hydrating the dry liposome-forming lipid or mixture of lipids with the solution of ISS-ODN to yield liposomal ISS-ODN.

As will be shown in the following specific Examples, the method of the invention provides a highly effective entrapment of the biologically active material in the liposomes, typically equal or greater than 60% (from the initial amount of ISS-ODN
20 employed by the method).

According to the present invention the lipids are preferably freeze dried, i.e. by lyophilization thereof, resulting in a powder with a unique arrangement of the lipids enabling the effective loading of the ISS-ODN upon hydration into liposomes formed by the hydration.

25 The lipids may be any substance that forms liposomes upon dispersion thereof in an aqueous medium. Preferred liposome-forming amphipathic substances are of natural sources, semi-synthetic or fully synthetic molecules; negatively or positively charged phospholipids, sphingolipids, or other lipids optionally combined with a sterol, such as cholesterol, and/or with lipopolymers, such as PEGylated lipids.

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The liposome-forming amphipathic substances may include saturated or unsaturated amphiphiles. Non-limiting examples of such amphiphiles are phospholipids including, without being limited thereto, fully hydrogenated, partially hydrogenated or non-hydrogenated soybean derived phospholipids, egg yolk phospholipids, dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG),
5 phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, sphingomyelins, and mixtures of the above.

A preferred phospholipid combination according to the invention includes a mixture of DMPC and DMPG at a molar ratio of DMPC:DMPG between about 1:20 and
10 20:1. Such mixtures may be combined with cholesterol, and/or PEGylated lipids. PEGylated lipids are commercially available. Preferred PEGylated lipids include, without being limited thereto, DSPE-PEG²⁰⁰⁰ [Haran, G., *et al. Biochim. Biophys. Acta* **1151**:201–215 (1993)] or dihexadecyl phosphatidyl PEG²⁰⁰⁰ (DHP-PEG²⁰⁰⁰) [Tirosh, O., *et al. Biophys. J.* **74**:1371–1379 (1998); US Patent No. 6,165,501]

15 The solvent according to the invention is any solvent with which lipids may solubilize or be dissolved. Such solvents include, *inter alia*, the water miscible polar solvent, tertiary-butanol and the water immiscible apolar solvent, cyclohexane.

According to the method of the invention it is advisable to keep the ISS-ODN in a medium isoosmotic to plasma such as 0.9% sodium chloride, or cryoprotectant, which is a
20 pharmaceutically acceptable agent such as lactose, sucrose or trehalose or NaCl/cryoprotectant mixture. Thus, the aqueous solution according to the present invention is a physiologically acceptable aqueous medium in which ISS-ODN can solubilize, dissolve, or disperse and may be selected from 0.9% NaCl by weight (Saline), buffered Saline such as phosphate-buffered Saline (PBS), 5% dextrose, buffered
25 dextrose, 10% sucrose and buffered sucrose, or any combination of the same. Alternatively, the ISS-ODN can solubilize in pyrogen-free sterile water (at times referred to as 'water for injection') and after hydration of the dry amphipathic substance, the resulting dispersion is adapted to the physiological conditions suitable for administration.

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According to a second aspect, there is provided by the present invention a combination of two compositions, including a first composition comprising dry liposome-forming lipids and a second composition comprising ISS-ODN, the combination is for use in the preparation of a pharmaceutical formulation comprising liposomal ISS-ODN, the preparation being in accordance with the method of the present invention.

The combination of the invention may be obtained in the form of a package. Accordingly, the present invention also concerns a combination at least one first composition of dry liposome-forming lipids; and at least one second composition of ISS-ODN (either dry or in an aqueous solution); in the form of a package further comprising instructions for use of the first and second compositions for the preparation of a pharmaceutical formulation, said instructions comprising hydrating the dry lipid(s) of said first composition with an aqueous solution of ISS-ODN to obtain liposomes loaded with said ISS-ODN (preferably more than 60%); and the package further comprising instructions prescribing administration of said pharmaceutical formulation to a subject in need thereof.

The dry liposome-forming lipids and the ISS-ODN are each contained in a separate vial. The package, according to the invention, may contain more than one of said first composition of dry lipid(s) in separate vials and more than one of second composition comprising ISS-ODN, the instructions for selection and use of the compositions will depend on the specific liposome/ISS-ODN formulation of interest. These instructions may be addressed to the physician, to the pharmacists or even to a subject of the treated individual.

The package may further comprise an aqueous medium, e.g. a physiologically acceptable aqueous medium, in which the ISS-ODN is dissolved or diluted. Alternatively, the aqueous medium may be obtained separately, as it is typically a commercially available medium. Selection of the medium suitable for use will depend on considerations known to those versed in the art and, therefore, do not need to be further discussed herein.

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According to one embodiment, the package comprises two or more compositions of dry lipids and optionally two or more compositions of ISS-ODN, thereby enabling to construct different pharmaceutical combinations according to the desired effect to be achieved and instructions prescribed by the medical practitioner. The package may be for use by the physician, by the pharmacist or, at times, by the subject in need of the liposomal formulation.

The invention also concerns pharmaceutical formulations comprising as active ingredient a therapeutically effective amount of liposomal ISS-ODN and optionally a pharmaceutically acceptable additive, the liposomal ISS-ODN being prepared by the method of the invention.

Alternatively, the package may contain at least: (a) one first composition of a dry liposome-forming lipid and (b) liposomal ISS-ODN prepared by the CO method. Mixture (b) may also be in a form of aqueous dispersion.

In fact, the pharmaceutical formulation of the invention is basically the liposomal formulation obtainable by the method of the invention but adapted for administration to the individual in need of a treatment or prevention of an identified disease.

The liposomal ISS-ODN is administered and dosed in accordance with good medical practice, taking into account the nature of the active ingredient, the clinical condition of the individual patient, the site, route and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners.

The liposomal ISS-ODN of the invention may be administered in various ways. It can be administered in combination with pharmaceutically acceptable diluents, excipients, additives and adjuvants, as known in the art, e.g. for the purposes of adding flavors, colors, lubrication or the like to the liposomal formulation.

The pharmaceutically acceptable diluent/s, excipient/s, additive/s employed according to the invention generally refer to inert, non-toxic substances which preferably do not react with the liposomal formulation of the present invention.

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Yet, since the ISS-ODN loaded in the liposomes are known to act as immunoadjuvants, the formulation of the invention may comprise other biologically active agents, such as antigens. The additional agents may be in a free form or also encapsulated in liposomes (in the same or different liposomes of the immunoadjuvant) and may be administered simultaneously, concomitant or within a predefined time interval from administration of the immunoadjuvant. Further, the antigen may be, *inter alia*, derived from a killed or modified (e.g. genetically) organism or virus.

The liposomal ISS-ODN can be administered orally, intranasally, or parenterally including intravenously, intraarterially, intramuscularly, intraperitoneally, subcutaneously, intradermally, and intrathecally, and by infusion techniques. Yet further, the liposomal ISS-ODN of the invention may be made into aerosol formulations for administration by inhalation. Such aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They also may be formulated as pharmaceuticals for non-pressured preparations, such as in a nebulizer or an atomizer. The manner of administration will depend on different considerations known to the man of the art, e.g. on the type of agent to be loaded into the liposome.

Finally, a method for the prevention or treatment of a disease is also provided by the present invention, the method including administration to a subject in need of liposomal ISS-ODN of the invention.

According to a preferred embodiment, the dosage for the liposomal ISS-ODN include up to 2,000 mg of loaded liposomal vesicles, measured by lipid, per kg body weight of the treatment subject. It should be noted, however, that the accurate dosage can vary dramatically, the variation depends on e.g. the type and efficacy of the ISS-ODN entrapped by the liposome, the efficiency of encapsulation (albeit being high with the method of the invention), the route of administration, the disease to be treated and the like. The respective parameters may be easily optimized by those skilled in the art and can thus be regarded as being routine experiments.

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The invention will now be further explained by the following non-limiting examples. While the foregoing description describes in detail only a few specific embodiments of the invention, it will be understood by those skilled in the art that the invention is not limited thereto and that other variations in form and details may be possible without departing from the scope and spirit of the invention as defined by the claims, which are to be read as included within the disclosure of the specification.

SPECIFIC EXAMPLES

General

ISS-ODN-loaded liposomes

10 The lipids employed for liposome formation in the following examples were DMPC and DMPG, with or without cholesterol. Two types of liposomes containing CpG were formed, for the purpose of comparison of the method of preparation of the present invention with another hitherto known method. The two encapsulation methods employed are designated herein as *post encapsulation* (the method of the present invention) and *co-encapsulation*.

1. ***Post encapsulation:*** A lyophilized mixture of the lipids was hydrated with the ISS-ODN, *a priori* dissolved in pure water, and the liposomal preparation was diluted in 0.9% NaCl (Saline) or phosphate-buffered Saline (PBS).
- 20 2. ***Co-encapsulation:*** The solubilized lipids in tertiary butanol and ISS-ODN in pure water were co-lyophilized overnight and then hydrated with 0.9% Saline.

Materials and Reagents

Influenza subunit vaccines (HN) – Subunit preparations containing mainly the influenza viral surface proteins hemagglutinin (H) and neuraminidase (N), 80-90% and 5-10% (w/w), respectively, derived from strains A/Sydney/5/97 (H3N2), A/Beijing/262/95 (H1N1), A/New Caledonia/20/99 (H1N1), A/Panama/2007/99

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(H3N2), and B/Yamanashi/166/98, were provided by Dr's. R. Glück and R. Zurbriggen, Berna Biotech, Bern, Switzerland.

Hepatitis B vaccines (Hb) – Recombinant, yeast (*Hansenula polymorpha*) derived hepatitis B's antigen (HBsAg, Rhein Biotech, Dusseldorf, Germany, Diminsky
5 *et al.* 1997).

Tuberculosis vaccine –Combined vaccines composed of *M. tuberculosis* derived recombinant proteins (ESAT-6, L7/L12 and 85 B, or Sod, 85B and CFP21) mixed with Ribi adjuvant (Sigma, USA), or without Ribi adjuvant.

Dimyristoyl phosphatidylcholine (DMPC) - Lipoid PC 14:0/14:0 562157
10 (Lipoid GmbH, Ludwigshafen, Germany).

Dimyristoyl phosphatidylglycerol (DMPG) - Lipoid PG 14:0/14:0 602035-1 (Lipoid GmbH, Ludwigshafen, Germany).

ODN - Endotoxin-free (1<ng/mg DNA) phosphorothioate ISS-ODN (ODN 1018), (5'-TGACTGTGAACGTTTCGAGATGA-3') and the "mutant" M-ODN,
15 lacking the CpG motif (ODN 1019), (5'-TGACTGTGAAGGTTAGAGATGA-3'), both dissolved in distilled water, were obtained from Trilink Biotechnologies, San Diego, CA, USA.

Two additional phosphorothioate ISS-ODNs were obtained from the Weizmann Institute, Rehovot, Israel, ODN 54076 (thioate): TCCATAACGTTGCAAAC-
20 GTTCTG, and ODN 51997 (thioate): TCCATGACGTTCTGACGTTCTG.

Methods of preparation

Preparation of soluble influenza HN antigen

The subunit preparations were used each separately or combined then diluted in sterile phosphate-buffered saline (PBS pH 7.4) for injection or intranasal
25 administration (0.15–1 µg protein/dose of each viral strain).

Preparation of Liposomal ISS-ODN (Lip ISS-ODN) and stability determination

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In the first series of experiments, ISS-ODN (ODN 1018) and M-ODN (ODN 1019) were encapsulated in large (mean diameter 1400 ± 200 nm) multilamellar vesicles (MLV) composed of DMPC and DMPG (DMPC:DMPG, 9:1 mole ratio), at a lipid:ODN ratio of 50:1-500:1 (w/w), under aseptic conditions as follows: The phospholipids were dissolved in tertiary butanol and freeze dried by lyophilization over night. The lipid powder (lipid cake) was then rehydrated at room temperature with the ODN solution. To ensure efficient encapsulation, soluble ODN was added in a minimal volume (e.g. for 10 mg-30mg lipid, 25-50 μ l of ODN solution was added). This was then vortexed vigorously for about 1 min. until a paste was obtained. The paste was then gradually diluted by further vortexing with sterile PBS or Saline to obtain the required concentration. This method corresponds to the **post encapsulation** method of the present invention.

To determine encapsulation efficiency, the liposomal preparation was centrifuged at 4°C, for 1hr. at 45,000 rpm. The liposome pellet and the supernatant (containing non-encapsulated ODN and traces of small liposomes) were subjected to a 2-phase lipid extraction procedure [Bligh, E.J. and Dyer, W.J. (1959) *Canadian J. Biochem. Physiol.* 37:911-917], and the amounts of free and encapsulated ODN and liposomal phospholipids were assessed by organic phosphorus determination [Barenholz, Y. and Amselem, S. (1993) in *Liposome technology*, 2nd ed., Vol I. (Gregoriadis G, ed.), CRC Press, Boca Raton, FL, pp. 501-525 (1993)].

Using the following ratios (w/w) of lipid:ISS-ODN – 50:1, 100:1, 300:1 and 500:1, the mean encapsulation efficiency (of 3 experiments) was 60, 75, 90 and 95%, respectively. No significant ODN leakage (<10%) from the liposomes was found after storage in aqueous dispersion for three months at 4°C. To avoid overloading the mice with extra lipids, which can cause nonspecific immune stimulation [Kedar, E., *et al. J. Immunother.* 23:131-145 (2000)], the formulation prepared at a 100:1 (w/w) lipid:ODN ratio (mean encapsulation efficiency, 75%) was chosen for vaccination experiments. The free/liposomal ISS-ODN and M-ODN were used at 5-25 μ g/mouse/dose.

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In a second series of experiments, the POST technique described above was compared to another procedure for encapsulating ISS-ODN in liposomes that were prepared by co-lyophilization of a mixture consisting of the solubilized lipids (in tertiary butanol) and ISS-ODN in aqueous solution, followed by hydration with 0.9% NaCl(referred to herein as the **co-encapsulation** method).

In these experiments (Table 1), ODN 1018 and the influenza antigens (HN) were encapsulated, each separately or combined, in liposomes composed of DMPC alone, DMPC/DMPG (9:1 molar ratio), or DMPC/cholesterol (6:4 molar ratio), using the post- or the co-encapsulation techniques at a lipid/ODN w/w ratio of 100/1, and at a lipid/protein(HN) w/w ratio of 300/1. Both techniques resulted in 60–80% encapsulation of ODN 1018 in all lipid compositions tested, when separate vesicles were used for ISS-ODN and the influenza antigens. For co-encapsulation of ODN 1018 and HN within the same vesicles, the CO technique was superior over the POST technique (70% vs 30% ODN 1018 encapsulation).

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Table 1-Encapsulation and co-encapsulation of influenza antigens (HN) and ISS-ODN 1018 in various liposomal formulations (MLV) prepared by the POST or the CO technique

Formulations	Agent	Encapsulation method for ODN	% HN encapsulation	% ODN encapsulation
DMPC				
Same vesicles	HN+ODN	POST	52-80	3-28
		CO	62	52-56
Separate vesicles	HN+ODN	POST	80-100	67-89
		CO	100	42-51
	HN alone	POST	87-93	-
	ODN alone	POST	-	62-63
		CO	-	61-66
DMPC/DMPG (9:1)				
Same vesicles	HN+ODN	POST	50-80	14-24
		CO	66	78-82
Separate vesicles	HN+ODN	POST	80-100	72-86
		CO	100	79-80
	HN alone	POST	80-100	-
	ODN alone	POST	-	61-66
		CO	-	66-67
DMPC/Chol (6:4)				
Same vesicles	HN+ODN	POST	NT	14-22
		CO	NT	69-77
Separate vesicles	HN+ODN	POST	NT	52-64
		CO	NT	63-82
	HN alone	POST	NT	-
	ODN alone	POST	-	64
		CO	-	63

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The data presented in Table 1 is a compiled summary of 3 separate experiments, using subunit vaccines derived from influenza A/Panama/2007/99 (H3N2) and B/Yamanashi/166/98 strains.

According to the POST-encapsulation method, the lipids dissolved in tertiary butanol and lyophilized overnight. The dried lipid powder was then hydrated by stepwise addition (in 25-50 μ l aliquotes) and vortexing of HN and/or ODN solution. According to the CO-encapsulation method, the solubilized lipids (solubilized in tertiary butanol as described above) are mixed with the ODN solution and co-lyophilized overnight, then hydrated stepwise (as above) with the HN solution or saline. Due to HN instability upon lyophilization, HN was encapsulated using the POST technique only. The lipid/ODN (w/w) ratio was 100/1, and the lipid/HN (w/w) ratio was 300/1.

Mixing of preformed empty liposomes (any of the formulations) in aqueous (saline) suspension with HN and ODN resulted in encapsulation (association) efficiency of 40-60% and 1-3%, respectively.

In a third series of experiments, an approximately 70% encapsulation was obtained at a lipid: ODN w/w ratio of 100:1 using ISS-ODN tagged with the fluorescent marker FITC. Liposomes were pelleted and extracted as above and the level of CpG encapsulation in liposomes was determined by two methods: organic phosphorus determination (described above) and from the FITC fluorescence intensity at 525 nm (excitation 495 nm).

In a fourth series of experiments, liposomal ISS-ODN was prepared at 100:1 and 300:1 lipid: ODN w/w ratios using saline or citrate buffer (containing 150 mM NaCl in 5 mM sodium citrate) pH 6.5-6.7 as final media for storage. The preparations were stored for up to 5 months at 4°C and at room temperature. ISS-ODN leakage and liposome lipid stability were tested 1, 3 and 5 months after preparation. The lipids in the dried lipid "cake" used to prepare the liposomal vaccine by the "post" encapsulation method were found to be chemically stable when stored at 4°C for at least 2 years.

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At a lipid: ODN w/w ratio of 100:1 there was a $\leq 5\%$ ODN leakage at 1 month and 5-15% leakage at 3 and 5 months when stored in unbuffered saline at 4°C and in citrate buffer at both 4°C and room temperature. Approximately 30% release was noted in the saline preparation at room temperature after 3 months. Thus, the liposomal
5 formulation as aqueous suspension is very stable (at least for 5 months at 4°C).

Liposome lipid stability was determined by thin layer chromatography (TLC) and by the release of non-esterified fatty acids (NEFA) as a result of aryl ester hydrolysis. No significant lipid degradation ($< 5\%$ hydrolysis) was noted up to 3 months at both temperatures for both saline and citrate buffer. A marked degradation ($> 10\%$) was found
10 at 5 months, but only in unbuffered saline at room temperature. Much lower level of degradation was found in saline/citrate buffer than in unbuffered saline. Formulations prepared at a lipid:ODN w/w ratio of 300:1 were slightly less stable and leakier than those prepared at a 100:1 ratio.

15 *Preparation of Liposomal HN (Lip HN)*

HN-loaded large multilamellar vesicle (MLV) liposomes (mean diameter, 1.5 μm) were prepared by using the **POST-encapsulation** method as described above in connection with preparation of Lip ISS-ODN by hydrating the dry lipid cake with the HN solution. In addition, HN was encapsulated with the ISS-ODN in the same vesicles
20 using the POST technique for HN and the POST or CO technique for ISS-ODN, as summarized above (Table 1).

In short, vials of 100 mg of DMPC:DMPG (9:1 molar ratio), DMPC alone, or DMPC/cholesterol (6:4 molar ratio) solubilized in tertiary-butanol, were frozen and then lyophilized over night to form the dry lipid cake. Upon need, the dry lipid was hydrated
25 with a mixture of the subunit (HN) preparations (derived from 1, 2, or 3 viral strains, see materials and reagents) by adding the soluble HN subunits at a lipid:HN ratio of 300:1 (w/w) in increments of 50 μl and vortexing vigorously. Liposomes co-loaded with HN and ISS-ODN 1018 were prepared by (a) hydration of the dry lipids, performed as above, with an aqueous solution mixture containing HN + ISS-ODN 1018 (the POST

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technique), (b) co-lyophilization of the lipid + ISS-ODN solution (CO), followed by hydration with the HN solution (POST). The liposomes were then further diluted in sterile saline or PBS and stored at 4°C.

Encapsulation efficiency was assessed by spinning the loaded liposomes at 4°C for 30 min. at 14,000 rpm (under these conditions free HN does not precipitate while the majority of the MLV liposomes do), and by determining the protein concentration of the supernatant and of the liposomal fraction using a modified Lowry determination assay [Peterson G.L., Methods Enzymol. 91:95-119 (1983)]. HN Encapsulation in separate vesicles or in vesicles also loaded with ISS-ODN was found to be 60-90%, depending on viral stain, using the two procedures described above. ODN encapsulation was determined as described above and it was 60-80% when encapsulated alone by both procedures or when encapsulated together with HN in the same liposomes using procedure (b) described above. ISS-ODN encapsulation was 20-30% when encapsulated together with HN using procedure (a) described above (Table 1).

For both Lip ISS-ODN and Lip HN, the lipid integrity was found to be very high (>95%) by analyzing samples of the lipids by thin layer chromatography (TLC).

Example 1 - Enhancement of the systemic anti-viral humoral response by liposomal ISS-ODN (Lip ISS-ODN) co-administered intramuscularly (i.m.) with influenza subunit vaccines

Female Balb/c mice, 6-8-weeks-old (5-6 per group) were vaccinated once (0.1 ml) i.m, with a divalent influenza subunit vaccine composed of the viral surface proteins hemagglutinin and neuraminidase (HN) derived from the A/Beijing/262/95-like (H1N1) and B/Yamanashi/166/98-like strains (0.15 µg protein of each strain). The HN antigens were given either in soluble form or entrapped in liposomes (Lip HN), alone or in combination with free ISS-ODN (ODN 1018) or Lip ISS-ODN (5, 12.5, 25 µg, Table 2). The liposomes comprised DMPC:DMPG (9:1 mole ratio), and HN and ISS-ODN were encapsulated by the POST technique. Preparation of the Lip HN and Lip ISS-ODN is described herein before.

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Two additional groups (group 9 and group 10, Table 2) were immunized with liposomes co-entrapping the HN antigens and the ISS-ODN. Mutant ODN (M-ODN) lacking the immunostimulatory sequence served as control. Sera were tested 3 weeks (21 days) and 3 months (90 days) postvaccination for hemagglutination-inhibiting (HI) Abs [Sever, J. (1962). *J Immunol.* 88:321-325] (Table 2) and for antigen-specific IgG1 and IgG2a Abs (Table 3). In the latter test, antigen-specific isotypes were tested 21 and 90 days postvaccination on pooled sera from each group (starting at a 1/10 dilution) by ELISA [Babai *et al.* Vaccine 17:1239-1250 (1999)]. The antibody (Table 3) titer is expressed as the reciprocal of the highest serum dilution yielding 50% of the maximum absorbance obtained with “standard” immune serum, after subtracting the control (antigen + normal mouse serum).

Table 2-The anti-hemagglutinin response (HI titer) following vaccination with free/liposomal divalent influenza vaccine

		HI titer (mean±SD) against:			
Vaccine and dose		A/Beijing (Day 21) ^a	B/Yamanashi (Day 21) ^a	A/Beijing (Day 90) ^a	B/Yamanashi (Day 90)
1.	HN 0.15 µg	20±14 (20%)	6±5 (0%)	128±44 (100%)	12±18 (20%)
2.	Lip HN	56±22 (100%)	24±17 (40%)	208±107 (100%)	48±30 (60%)
3.	HN+ISS-ODN 5µg	28±11 (40%)	20±14 (20%)	160±150 (80%)	26±31 (20%)
4.	HN+ISS-ODN 12.5 µg	24±17 (40%)	20±12 (20%)	44±36 (60%)	12±18 (20%)
5.	HN+ISS-ODN 25 µg	20±20 (40%)	16±9 (0%)	82±76 (60%)	18±13 (20%)
6.	HN+Lip ISS-ODN 5 µg ^b	128±44 (100%)	80±0 (100%)	352±175 (100%)	68±11 (100%)
7.	HN+Lip ISS-ODN 12.5 µg ^b	96±36 (100%)	72±18 (100%)	288±72 (100%)	96±61 (100%)
8.	HN+Lip ISS-ODN 25 µg ^{b,c}	176±88 (100%)	144±36 (100%)	480±225 (100%)	144±36 (100%)
9.	Lip (HN+ISS-ODN 5 µg)	160±0 (100%)	72±18 (100%)	448±175 (100%)	72±18 (100%)
10.	Lip (HN+ISS-ODN 25 µg)	144±36 (100%)	128±44 (100%)	448±175 (100%)	104±54 (100%)
11.	HN+M-ODN 25 µg	14±19 (20%)	8±8 (0%)	106±136 (60%)	8±11 (0%)
12.	HN+Lip M-ODN 25 µg	30±10 (40%)	22±11 (20%)	176±88 (100%)	20±14 (20%)

^a In parentheses, % seroconversion = % of mice achieving a HI titer ≥40.^b Groups 6, 7, 8 vs. groups 3, 4, 5, P <0.05 (t-test) for HI titer at both time points against the two viruses.^c Group 8 vs. group 12, P ≤0.01 for HI titer at both time points against the two viruses.

Table 3 - Anti-HN IgG isotypes following vaccination with a divalent influenza vaccine co-administered with free/liposomal ISS-ODN

Vaccine	Mean IgG titer against							
	A/Beijing d.21		B/Yamanashi d.21		A/Beijing d.90		B/Yamanashi d.90	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
1. HN 0.15 µg	<10	<10	20	<10	70	20	100	80
2. Lip HN	20	<10	80	<10	110	80	190	100
3. HN+ISS-ODN 5 µg	<10	70	<10	100	<10	350	30	450
4. HN+ISS-ODN 12.5 µg	<10	32	<10	80	<10	500	<10	380
5. HN+ISS-ODN 25 µg	<10	64	<10	70	<10	350	<10	450
6. HN+Lip ISS-ODN 5 µg	<10	450	<10	600	20	2500	<10	5000
7. HN+Lip ISS-ODN 12.5 µg	<10	560	<10	400	<10	2500	<10	4200
8. HN+Lip ISS-ODN 25 µg	<10	400	<10	600	<10	2400	<10	4600
9. Lip (HN+ISS-ODN 5 µg)	<10	400	<10	500	<10	2000	<10	4200
10. Lip (HN+ISS-ODN 25 µg)	<10	550	<10	800	10	1800	<10	5000
11. HN+M-ODN 25 µg	<10	<10	<10	<10	190	50	200	20
12. HN+Lip M-ODN 25 µg	<10	<10	<10	<10	300	20	300	10

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Table 2 shows the results of hemagglutination inhibition (HI), while Table 3 shows the results of the ELISA of antigen-specific IgG1 and IgG2a Abs.

As can be seen in Table 2, the HI titer, as well as the percent seroconversion, of mice co-immunized with Lip ISS-ODN (groups 6-8) were significantly greater than those of mice vaccinated with antigen alone (groups 1, 2) or with antigen combined with soluble ISS-ODN (groups 3-5). The HI titers obtained with Lip ISS-ODN were 2-8-fold higher than with soluble ISS-ODN. At 3 weeks postvaccination, the seroconversion (HI titer ≥ 40) rate was 0-40% using soluble ISS-ODN or M-ODN and 100% with Lip ISS-ODN. The superior adjuvant activity of Lip ISS-ODN was seen at all doses, at both time points, and for the two viral strains. The response attained at the lowest ISS-ODN dose (5 μ g) was similar to that obtained with the highest dose (25 μ g). Vaccination with liposomes loaded with the antigen and the ISS-ODN in the same vesicles (groups 9, 10) (using the post technique for both) produced a response similar to that achieved with a mixture of Lip ISS-ODN and the free antigen (groups 6-8). However, it is worth noting that under such conditions (i.e., mixing of preformed liposomes with free HN) approximately 40% of the antigen became liposome associated. Similar results were also obtained with a mixture of Lip ISS-ODN and Lip HN (data not shown). M-ODN, free and liposomal, had no adjuvant effect (groups 11, 12), indicating the need for the CpG sequence for the adjuvant activity.

Assessment of antigen-specific IgG1 and IgG2a (Table 3) showed a modest increment of IgG2a (and abrogation of IgG1) by soluble ISS-ODN (groups 3-5) and a dramatic increase in IgG2a by Lip ISS-ODN either mixed with free antigen (groups 6-8) or co-entrapped with the antigen (groups 9, 10). The IgG2a levels produced by Lip ISS-ODN were 4-18 times greater than with soluble ISS-ODN. M-ODN, free and liposomal, slightly increased the IgG1 level and reduced the IgG2a level at 3 months post-vaccination (groups 11, 12).

Thus, a single vaccine dose containing even 5 μ g of Lip ISS-ODN results in very high HI titers and specific IgG2a levels, with no IgG1 response, namely a Th1 type. This enhanced response lasts for at least 3 months. The results of this experiment were reproduced in two additional experiments, using divalent and trivalent (adding the

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A/Sydney/5-97-like, H3N2, to the divalent preparation) vaccines. The HI titers on days 21-90 were 4-10-fold higher with Lip ISS-ODN (10 µg) than with non-liposomal ISS-ODN. Moreover, Lip ISS-ODN 1018 prepared by the POST technique and Lip ISS-ODN prepared by the CO technique had an equal adjuvant activity following intramuscular co-administration with the influenza HN antigens (data not shown).

In addition to testing the primary response, some groups (groups 5, 6) received a second vaccine dose 3 months after the primary dose (i.e. vaccination on days 0 and 90) and sera were tested 7 days later. The results of this additional test are presented in the following Table 4.

Again, as seen in the primary response, repeated co-vaccination with Lip ISS-ODN produced HI titers 3.3-5 greater and IgG2a levels 3.3-8 higher than with soluble ISS-ODN. No increase in IgG1 was seen with either formulation. These results indicate that Lip ISS-ODN preserves its adjuvant activity also with repeated administration.

Table 4- Secondary immune response of mice immunized with free/liposomal divalent influenza vaccine

Vaccine	HI titer (mean \pm SD) against:		Mean IgG titer against:			
	A/Beijing	B/Yamanashi	A/Beijing	B/Yamanashi	IgG1	IgG2a
HN 0.15 μ g	320 \pm 0 (100%)	260 \pm 150 (100%)	500	<10	1000	200
HN+ISS-ODN 25 μ g	896 \pm 350 (100%)	368 \pm 260 (100%)	400	1,000	600	3,000
HN+Lip ISS-ODN 25 μ g	3000 \pm 1500 (100%)	1900 \pm 800 (100%)	600	8,000	1,000	10,000
HN+M-ODN 25 μ g	640 \pm 0 (100%)	170 \pm 200 (50%)	500	<10	800	100

Induction of DTH response by Lip ISS-ODN 1018

In addition to assessing the systemic humoral response, some groups that were vaccinated twice at a 3-month interval were tested for DTH response following subcutaneous (s.c.) injection of hemagglutinin. In particular, 7 days after the second vaccine dose 10 µg of A/Beijing hemagglutinin was injected s.c. into the hind footpad, and footpad swelling was measured at 24 hrs. The increase in footpad thickness (mm) was determined by comparison to the footpad thickness prior to hemagglutinin injection. As control, the contralateral foot was injected with PBS (increase in footpad thickness at 24 hrs. for PBS was 0.037±0.05 mm). The results are shown in the following Table 5.

10

Table 5-DTH response following vaccination with a divalent influenza vaccine co-administered with free/liposomal ISS-ODN

Vaccine	Increase in footpad thickness (mm) ^a	Incidence of mice with DTH ^b
	Mean ±SD	
1. Lip HN	0.06±0.01	0/3
2. HN+ISS-ODN 5µg	0.12±0.08	2/5
3. HN+Lip ISS-ODN 5 µg	0.16±0.09	2/5
4. Lip (HN+ISS-ODN 5 µg)	0.52±0.2	5/5
5. HN+M-ODN 25µg	0.04±0.03	0/3

^a P values: group 4 vs. all groups ≤ 0.004.; group 2 vs. group 3 >0.05 (t-test).

15 ^b An increase in footpad thickness greater than 10%, relative to time 0, was considered a positive DTH response.

As can be seen from Table 5, a significant DTH response in all mice (5/5) was observed in group 4 vaccinated with liposomes encapsulating both the antigen and the ISS-ODN (5 µg). Mice immunized with soluble antigen mixed with 5 µg of soluble or liposomal ISS-ODN (groups 2,3) developed a moderate response, and only 2/5 were considered positive. Vaccination with antigen alone or with antigen coadministered with M-ODN (25 µg) did not induce a response.

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DISCUSSION

The above results demonstrate that ISS-ODN 1018 can be encapsulated in large multilamellar liposomes with high efficiency (up to 95%) using the "post" technique, and that the liposomal formulation is a considerably more potent parenteral adjuvant in mice than the soluble form of ISS-ODN.

A single dose of 5 µg Lip ISS-ODN was by far more potent (4-11 times, Tables 2,3) for the two viral strains than 25 µg of free ISS-ODN. The enhanced potency was reflected in both humoral and cellular (DTH) responses. As with free ISS-ODN, Lip ISS-ODN mainly enhanced Th1-biased immunity, based on the relative IgG1 and IgG2a levels upon i.m. administration.

Further, the results disclosed herein and additional results (data not shown) show that the same increment in the humoral response was obtained when free or liposomal antigen (HN) was administered together i.m. with Lip ISS-ODN (in separate liposomes), and when the vaccine consisted of antigen and ISS-ODN entrapped together in the same liposomes using the post technique. These results differ from those reported recently by two groups on liposomal plasmid DNA and liposomal ISS-ODN. Gursel et al. [Gürsel, M., et al. (1999) *Vaccine*. 17:1376-1383] found that the adjuvant activity of a non-coding plasmid was demonstrated only when the plasmid and the hepatitis B surface antigen were entrapped together in the same liposomes, but not in separate liposomes. Ludewig et al. [Ludewig, B., et al. (2001) *Vaccine*. 19:23-32] showed that the anti-viral and anti-tumor responses following vaccination with liposome-entrapped peptides could be augmented by ISS-ODN. However, the response obtained with liposomal antigens admixed with non-liposomal ISS-ODN was similar to that produced by antigens and ISS-ODN incorporated into the same liposomes. This is not the case in our study; our data (Tables 2,3) show that ISS-ODN was an equally effective adjuvant (for the humoral response) when entrapped in the same liposomes with the antigen or in separate liposomes, and Lip ISS-ODN was always more effective than free ISS-ODN (5-25 µg). These differences appears to result from (a) the different and more efficient procedure of the present invention for the encapsulation and delivery of ISS-ODN, and the different chemical composition and size of the liposomes, both of which may affect the

localization of the ISS-ODN in the liposome and its delivery; and maybe also from (b) differences in the experimental systems, including the antigens and the type of ISS-ODNs, the route of administration, the dosage, and the assays used to monitor the response.

5 It should be noted, however, that in contrast to the effect of Lip ISS-ODN on the systemic humoral response, the DTH response (Table 5) was greater when the antigen and the ISS-ODN were encapsulated together in the same liposomes, as compared with the DTH response obtained with liposomal antigen mixed with liposomal ISS-ODN (in separate liposomes).

10 The superior adjuvant activity of Lip ISS-ODN over free ISS-ODN for intramuscular influenza vaccine was not limited to ODN 1018. In another experiment ISS-ODN 1018 was compared with two other ISS-ODNs containing CpG motifs. All three ISS-ODNs exhibited a similar adjuvant activity, and Lip ISS-ODN was 2-7 times more potent (for HI titer and IgG2a titer) than the corresponding free ISS-ODN
15 (Table 6). According to this specific experiment, BALB/c mice (n=4, group 7, 3 mice/group) were injected once i.m. with 0.5 µg of a subunit vaccine derived from influenza A/New Caledonia/20/99 (H1N1), alone and combined with 10 µg of free or liposomal ISS-ODN. Hemagglutination inhibition (HI) was tested on individual sera and Ig were tested by ELISA on pooled sera. The liposomes (MLV) were composed of
20 DMPC/DMPG (9:1 mole ratio) and ODN was encapsulated by the POST technique at a 100/1 lipid/ODN w/w ratio.

Table 6-Comparison of different ISS-ODNs as adjuvants for influenza vaccine: HI, IgG1 and IgG2a titers 4 weeks post vaccination

Vaccine	Mean HI titer ^a	Mean IgG1 titer	Mean IgG2a titer	IgG2a/IgG1 ratio
1. None	5 (0)	<10	<10	-
2. HN alone	9 (0)	1500	60	0.04
3. HN + free ODN 1 ^b	52 (75)	900	1500	1.7
4. HN + lip ODN 1	140 (100)	2000	2800	1.4
5. HN + free ODN 2 ^c	31 (50)	45	700	15.5
6. HN + lip ODN 2	210 (100)	1500	3500	2.3
7. HN + free ODN 3 ^d	57 (33)	65	4500	69.2
8. HN + lip ODN 3	180 (100)	2800	11000	3.9

^a In parenthesis, % seroconversion (% of mice with an HI titer ≥ 40).

^b ODN 54076 (thioate);

5 ^c ODN 51997 (thioate);

^d ODN 1018 (thioate).

Enhancement of mucosal anti-viral response and protective immunity by Lip ISS-ODN co-administered intranasally (i.n.) with influenza vaccine

A liposomal divalent vaccine (Lip HN, 0.5 μ g of each strain) was administered
 10 twice i.n., spaced 1 week, alone and in combination with free or Lip ISS-ODN (10 μ g),
 or with free cholera toxin (CT, 1 μ g), considered a powerful (yet toxic) mucosal
 adjuvant. In this case, HN and ISS-ODN were incorporated into the same DMPC/DMPG
 liposomes by the POST technique. Serum, nasal wash and lung homogenate were tested
 for antigen-specific IgG1, IgG2a and IgA Abs four weeks after the second vaccine dose,
 15 and protective immunity to viral challenge was assessed six weeks post-vaccination by
 determining lung virus titer. The mean Ig titer was tested by ELISA on pooled samples.
 Values obtained with normal mouse serum were subtracted. Lungs were washed x3 in
 cold PBS then homogenized in 1.5 ml PBS per lungs of each mouse (referred to as 1/10
 dilution). The homogenates were then centrifuged at 3000 rpm for 30 min at 4°C, the
 20 supernatants were collected and centrifuged at 14,000 rpm for 20 min at 4°C, and

antibody titer in the supernatants was measured. Nasal wash was made in 0.2 ml PBS (referred to as 1/10 dilution). Serial 2-fold dilutions were tested, starting with 1/20 sample dilution. The results are shown in the following Table 7. The ELISA antibody titer was determined as described for Table 3.

**Table 7 -Serum and mucosal anti-HN IgG1, IgG2a and IgA antibodies 4 weeks following intranasal vaccination with a liposomal
divalent influenza vaccine co-administered with free/liposomal ISS-ODN**

Vaccine (n = 5/group)	Location	Mean titer against					
		A/Beijing		B/Yamanashi			
		IgG1	IgG2a	IgA	IgG1	IgG2a	IgA
Lip HN	Serum	<20	<20	<20	<20	<20	<20
Lip HN+free ISS-ODN		<20	<20	<20	<20	40	<20
Lip (HN+ISS-ODN)		45	65	<20	100	300	90
Lip HN+CT		30	75	<20	300	100	50
Lip HN	Nasal wash	<20	<20	<20	<20	<20	<20
Lip HN+free ISS-ODN		<20	<20	<20	<20	<20	<20
Lip (HN+ISS-ODN)		90	55	110	90	60	60
Lip HN+CT		275	20	20	150	50	50
Lip HN	Lung homogenate	<20	<20	<20	<20	<20	25
Lip HN+free ISS-ODN		<20	<20	<20	<20	<20	<20
Lip (HN+ISS-ODN)		<20	70	200	<20	20	60
Lip HN+CT		475	55	200	350	75	20

As shown in Table 7, soluble ISS-ODN had no adjuvant effect (except for a low increase in serum IgG2a against one viral strain). In contrast, Lip ISS-ODN induced significant levels of antigen-specific IgG1, IgG2a and IgA in serum, nasal wash and lung. The response to the two viral strains included in the vaccine varied, however. Lip ISS-ODN and CT elicited similar levels of serum and mucosal IgG2a and IgA; the latter inducing higher levels of IgG1 as well. Thus, under the experimental conditions used, Lip ISS-ODN, but not soluble ISS-ODN, is capable of boosting both systemic and mucosal antigen-specific IgG2a and IgA Abs.

The remaining mice of this experiment (5 per group) were challenged i.n. with a recombinant A/Beijing virus. In particular, six weeks after the second vaccine dose, mice were lightly anesthetized with Halotane and 25 μ l live virus suspension per nostril was administered. The test virus was the reassortant virus X-127 (A-Beijing/262/95 (H1N1) x X-31 (A/Hong Kong/1/68 x A/PR/8/34), which is infectious to mice, 10^7 EID 50 (egg-infectious dose 50%). The mice were sacrificed on day 4 post-infection, the lungs were removed, washed x 3 in cold PBS, and homogenates in PBS of each group were pooled and centrifuged at 2000 rpm for 30 min. at 4°C and the supernatants collected. Serial ten-fold dilutions were performed and 0.2 ml of each dilution was injected in duplicate into the allantoic sac of 10-11 days-old fertilized chicken eggs. After 48 hrs., at 37°C the eggs were stored overnight at 4°C. Then 0.1 ml of allantoic fluid was removed and checked for viral presence by hemagglutination with chicken erythrocytes (0.1 ml of 0.5% suspension) for 30 min at room temperature. The lung virus titer is determined as the highest dilution of lung homogenate producing virus in the allantoic fluid (positive hemagglutination). A titer of 0.5 indicates 1 egg was positive and 1 egg was negative at the highest dilution.

Table 8-Protection against influenza virus infection 6 weeks following intranasal vaccination with a divalent influenza vaccine co-administered with free/liposomal ISS-ODN

Vaccine (n=5)	Lung virus titer (log10)
Unimmunized	6
Lip HN	5
Lip HN + free ISS-ODN	4
Lip (HN+ISS-ODN)	2.5
Lip HN + CT	3

5 Table 8 shows that, as compared with unimmunized mice, the lung virus titer of mice vaccinated with Lip HN alone, Lip HN+free ISS-ODN and Lip (HN+ISS-ODN) was reduced by 1, 2 and 3.5 logs, respectively (and 3 logs with CT). The 30-fold difference in lung virus titer between free and liposomal ISS-ODN is in good agreement with the differences seen in HI, IgG2a and IgA titers between these groups.

10 In another intranasal experiment (Table 9), a comparison was made between Lip ISS-ODN 1018 encapsulated by the POST technique and Lip ISS-ODN 1018 encapsulated by the CO technique. According to this experiment, BALB/c mice (n=5) were vaccinated i.n. on days 0, 7 with a subunit vaccine derived from influenza A/New Caledonia/20/99 (H1N1). In groups 6,7 separate vesicles were formed for HN and ODN 1018;
 15 in groups 8, 9 HN and ODN 1018 were encapsulated together within the same vesicles. In all formulations, HN was encapsulated by the POST technique. Liposomes (MLV) consisted of DMPC/DMPG (9:1 mole ratio). Response was measured 4 weeks after the second vaccine dose. CT-cholera toxin.

In most cases (Table 9, groups 6-9) both liposomal ISS-ODN formulations
 20 showed a similar adjuvant activity, except for the superior activity of Lip ISS-ODN formed by the CO technique upon administration together with free HN (groups 4,5). Moreover, Lip ISS-ODN was superior to free ISS-ODN with regard to HI and IgG2a titers.

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Table 9-The anti-influenza humoral response of BALB/c mice vaccinated intranasally with various liposomal formulations of HN and ISS-ODN 1018

Vaccine	Encapsulation method of ODN	Mean HI titer ^a	Mean IgG1 titer ^b	Mean IgG2a titer ^c
1. None	-	<10 (0)	<10	<10
2. HN 1 µg	-	<10 (0)	15	<10
3. HN+free ODN 10 µg	-	<10 (0)	400	100
4. HN+lip ODN	POST	<10 (0)	190	210
5. HN+lip ODN	CO	27 (60)	550	350
6. Lip HN +lip ODN	POST	26 (60)	1100	160
7. Lip HN +lip ODN	CO	12 (20)	1000	300
8. Lip (HN+ODN)	POST	24 (40)	350	300
9. Lip (HN+ODN)	CO	22 (40)	650	300
10. HN+CT 1µg	-	120 (100)	25000	400

^aTested by hemagglutination inhibition (HI) on individual sera. In parenthesis, % seroconversion (% of mice with an HI titer ≥ 40).

5 ^bTested by ELISA on pooled sera. The titer was determined as the highest serum dilution yielding OD=0.2.

In an additional experiment both the humoral and cellular responses were tested in parallel following parenteral vaccination with a subunit influenza vaccine co-administered with free or liposomal (lip) ISS-ODN.

BALB/c mice were immunized once intramuscularly (i.m.) with a monovalent
5 subunit vaccine (0.5 μ g) derived from A/New Caledonia/20/99-like (H1N1), alone and combined with 10 μ g of free or lip ISS-ODN (MLV liposomes (1.5 μ m) consisted of DMPC:DMPG at 9:1 mole ratio, lipid:ODN w/w ratio of 100:1 and prepared as described above). Mice injected with lip ISS-ODN alone served as control. Humoral (hemagglutination inhibition [HI] titer, serum and lung IgG1, IgG2a, IgA and IgE titer,
10 Table 10) and cellular (proliferation, cytokine production, cytotoxicity, Table 11) responses were assessed 4 and 6 weeks post-vaccination using spleen cells.

For determining cellular response, cells (0.5×10^6 /well) were incubated in U-shaped 96-well plates, in triplicate, with or without the HN antigen (0.5 μ g/well), in a final volume of 0.2 ml of RPMI 1640 medium supplemented with 5% FCS. After 72 h,
15 cultures were pulsed with 1 μ C 3 H-thymidine for 16 h.

For determination of IFN γ production cells, (2.5×10^6 /well) were incubated in 24-well plates, in duplicate, with or without the HN antigen (10 μ g/well), in a final volume of 1 ml of RPMI 1640 + FCS. Supernatants were collected after 72 h and tested by ELISA for murine IFN γ and IL-4.

20 For determining cytotoxic activity, responding splenocytes, (2.5×10^6 /well) were incubated in 24-well plates, in duplicate, with or without the HN antigen (10 μ g/well) in a final volume of 1 ml of RPMI 1640 + 10% FCS + 5×10^{-5} M 2-mercaptoethanol. On day 7 cytotoxicity was tested in a 4 h 51 Cr release assay (at an effector/target cell ratio of 15/1, in triplicate) against labeled non-infected P815 cells and P815 cells infected with
25 the reassortant virus X-127 (H1N1, which is infectious to mouse cells and cross-reactive with A/New Caledonia), using 0.2 ml of virus-containing allantoic fluid for 5×10^6 cells, for 2 h at 37°C. Specific cytotoxicity was calculated after subtracting the cytotoxicity against non-infected P815.

Table 10-The humoral response of BALB/c mice immunized i.m. with a monovalent influenza subunit vaccine, alone and combined with free/liposomal ISS-ODN

HN vaccine ^a (n=4)	Serum	Serum	IgG2a	Lung homogenate	
	HI titer	IgG1 titer	IgG2 titer	IgG1 titer	IgG2a titer
	(Mean \pm SD) at 4 weeks ^b	(Mean) at 6 weeks ^c	(Mean) at 6 weeks ^c	(Mean) at 6 weeks ^c	(Mean) at 6 weeks ^c
1. None	<10 (0)	<10	<10	<10	<10
2. HN (0.5 μ g) alone	<10 (0)	600	<10	75	<10
3. HN + ISS-ODN (10 μ g)	80 \pm 0 (100)	150	150	<10	25
4. HN + lip ISS-ODN (10 μ g)	320 \pm 113 (100)	300	1500	<10	150
5. Lip ISS-ODN alone	<10 (0)	<10	<10	<10	<10

^a prepared as described;

^b Mice were tested individually, in parentheses, % seroconversion (% of mice with HI titer \geq 40);

^c Tested by ELISA on pooled samples starting at 1/10 dilution. The reciprocal of the highest sample dilution yielding 50% of the maximum absorbance obtained with a "standard" immune serum was considered the IgG1 or IgG2a titer.

Table 11-The cellular response of BALB/c mice immunized i.m. with a monovalent influenza subunit vaccine, alone and combined with free/liposomal ISS-ODN

Vaccine ^a (n=4)	Proliferative response (stimulation index) ^b	IFN γ production (pg/ml)	% Specific cytotoxicity (E/T ratio = 15/1) ^{cm}
1. None	1.7	<10	2
2. HN (0.5 μ g) alone	3.1	60	9
3. HN + ISS-ODN (10 μ g)	2.2	50	7
4. HN + Lip ISS-ODN (10 μ g)	9.3	220	37
5. HN + Lip M-ODN (10 μ g)	4.3	20	6
6. Lip ISS-ODN alone	1.0	<10	6

^a Vaccine prepared as described above;

^b Stimulation index = mean cpm of cells cultured with antigen/mean cpm of cells cultured without antigen;

^c The values presented were obtained in antigen-containing cultures; no significant cytotoxicity (<5%) was seen in cells cultured without antigen. Cytotoxicity against non-infected p815 target cells was subtracted.

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As can be seen in Table 10, no HI response was induced at 4 weeks by antigen alone (HN, hemagglutinin + neuraminidase) or by lip ISS-ODN alone. Mice co-immunized with HN and free/lip ISS-ODN showed a 100% seroconversion (HI titer \geq 40); however, the HI titer obtained with lip ISS-ODN was 4 times greater than that
5 with free ISS-ODN. Testing by ELISA of antigen-specific immunoglobulin isotypes in serum and lung homogenates at 6 weeks post-vaccination, again showed no response to antigen alone and a moderate increase of IgG2a by co-administered free ISS-ODN. Using lip ISS-ODN as an adjuvant, the serum and lung IgG2a levels were 10 and 6 times higher, respectively, than those achieved with free ISS-ODN.

10 The presence of a relatively high level of IgG2a antibodies in the lung following parenteral vaccination with lip ISS-ODN is of particular interest. Lip ISS-ODN alone had no effect. No increase in IgG1 was seen with either formulation of ISS-ODN. Antigen-specific IgA and IgE were undetectable. Thus, for enhancing the humoral response, lip ISS-ODN (10 μ g) is 4-10 times more efficient than free ISS-ODN.

15 With regard to cellular responses (Table 11), free ISS-ODN had no adjuvant activity in any of the three tests performed. IFN γ (\geq 20 pg/ml) was detected only in cultures containing the antigen. No IL-4 (<20 pg/ml) was detected in any of the groups. As compared with free ISS-ODN, *in vitro* stimulated splenocytes of mice co-immunized with antigen and lip ISS-ODN exhibited a 4.2-, 4.4- and 5.3-fold greater
20 proliferative response, interferon γ production and specific cytotoxic activity, respectively. As control, stimulation of naive splenocytes with concanavalin A (2.5 μ g/well) produced 2000 pg/ml of IFN γ and 70 pg/ml of IL-4 (data not shown). This experiment indicates that, in addition to its superior adjuvant activity for humoral responses, lip ISS-ODN is also a more potent adjuvant (at least 4-fold under the
25 experimental conditions used) than free ISS-ODN for cellular responses in this model system.

Based on the IgG2a/IgG1 ratio (\geq 5, Table 10) and the induction of cellular responses (Table 11), lip ISS-ODN triggers a much stronger Th1-dominant response than free ISS-ODN.

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In an additional experiment the results of which are shown in the following Table 12, a comparison was made between free/lip ISS-ODN (1018), free/lip M-ODN (1019) and free/lip recombinant human IL-2 (previously used by us as an adjuvant in this model) as adjuvants for the A/New Caledonia subunit vaccine. In general, mice were immunized i.m. as detailed above and pooled sera were tested by ELISA 3 and 8 weeks post-vaccination. Antigen-specific IgG titers were calculated as detailed in connection with Table 3. Empty liposomes alone were not tested.

Table 12 - Antigen-specific serum isotypes following intramuscular vaccination of BALB/c mice with a monovalent influenza subunit vaccine, alone and combined with free/liposomal ISS-ODN or M-ODN

Vaccine (n=5)	IgG1 titer		IgG2a titer		IgG2a/IgG1 ratio	
	3W	8W	3W	8W	3W	8W
1. HN alone 0.5 µg	200	350	<10	<10	<0.05	<0.03
2. HN + ISS-ODN 10 µg	180	300	100	250	0.56	0.83
3. HN + lip ISS-ODN 10 µg	150	400	450	500	3.0	1.25
4. HN + M-ODN 10 µg	250	800	<10	50	<0.04	0.06
5. HN + lip M-ODN 10 µg	750	2000	<10	80	<0.013	0.04
6. HN + IL-2 10 µg	300	500	<10	35	<0.03	0.07
7. HN + lip IL-2 10 µg	750	6000	150	300	0.2	0.05

Both free and lip ISS-ODN markedly enhanced only the IgG2 level, the latter being 2-4.5 times more potent. In contrast, M-ODN, particularly lip M-ODN, enhanced mainly the IgG1 response (3-6-fold vs. antigen alone). Lip IL-2 increased both isotypes, particularly IgG1. This experiment demonstrates that the type of adjuvant markedly affects the type of response: lip ISS-ODN (Th1), lip M-ODN (Th2) and lip IL-2 (Th1+Th2).

In conclusion, this series of experiments indicated the superior adjuvant activity of liposomal ISS-ODN over free ISS-ODN for influenza vaccines administered i.m. or

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i.n. The stronger humoral and cellular responses elicited by Lip ISS-ODN correlated well with the greater protection against viral infection.

Furthermore, no toxicity (no change in body weight, breathing, mobility, fur) was apparent in mice immunized with Lip ISS-ODN once or twice, i.n. and i.m. No swelling or ulceration was noticed at the i.m. injection site.

Example 2 - Enhancement of the systemic anti-viral humoral response and cellular response by liposomal ISS-ODN 1018 (Lip ISS-ODN) co-administered with Hepatitis B vaccine

In addition to the influenza vaccines, the adjuvant activity of free and liposomal (lip) ISS-ODN 1018 with a recombinant (yeast-derived) hepatitis B surface antigen (HBsAg) particles, which serves as standard hepatitis B vaccine in humans, was tested in mice. In this experiment, BALB/c mice (females, 5-weeks old) were vaccinated i.m. with 0.2 µg (0.03 ml) of recombinant HBsAg (Rhein Biotech, Dusseldorf, Germany) on days 0 and 21, using naked antigen (the HBsAg particles alone) or "alum"-adsorbed antigen, (alhydrogel 2%, aluminum hydroxide gel adjuvant, Superfros Biosector, Frederikssund, Denmark, used as the human vaccine, with an alum/antigen w/w ratio of 25:1), each alone and combined with free/lip ISS-ODN (10 µg, DMPC/DMPG 9:1 mole ratio, lipid:ODN w/w ratio of 100:1). As controls, the antigen was co-administered with lip M-ODN (lacking the CpG motif) or empty liposomes.

For assessment of the humoral response (Table 13) (antigen-specific IgG1 and IgG2a antibodies, and HBsAg neutralizing antibodies) animals were bled on days 21 (for primary response) and 35 (for secondary response). Antigen-specific IgG isotypes were tested by ELISA, starting at a 1/10 serum dilution (as described in connection with the influenza model detailed above). The reciprocal of the highest serum dilution yielding OD = 0.2 (after subtracting OD with antigen + normal serum) was considered the IgG1 and IgG2a titer. Specific, neutralizing anti-HBs antibodies were measured using a commercial microparticle enzyme immunoassay (IMx AUSAB, Abbott Laboratories, USA) on serum samples diluted 1/2-1/10.

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The cellular response (proliferation, cytokine production, cytotoxicity) was tested on day 56 using pooled splenocytes of each group. The results are presented in the following Table 14. For IFN γ and IL-4 production, cells (5×10^6 /well) were incubated in duplicate in 24-well plates in a final volume of 1 ml of complete DMEM medium + 5% FCS, alone and together 5 μ g of HBsAg. Supernatants were collected after 48 h and IFN γ and IL-4 were tested by ELISA, using the Opt EIA Set (Pharmingen, USA). In parentheses, cytokine levels in non-stimulated cultures (incubated without antigen). For determining the specific cytotoxicity cells were incubated as above, using complete DMEM medium + 10% FCS + 5×10^{-5} M 2-mercaptoethanol and cytotoxicity was measured on day 6 (4 h 51 Cr release assay, 4000 target cells/well, in triplicate) against labeled non-transfected P815 and HBsAg-transfected P815 (generously provided by Dr. Joerg Reimann, University of Ulm, Germany), at effector/target (E/T) cell ratios of 50/1 and 5/1. Specific cytotoxicity was calculated after subtracting cytotoxicity against non-transfected P815. No significant specific cytotoxicity was obtained in spleen cells cultured without the antigen.

For determination of proliferation, cultures were carried out, in triplicate, in U-shaped 96-well plates, using 5×10^5 cells/well in a final volume of 0.2 ml of complete DMEM medium + 5% FCS + 5×10^{-5} M 2-mercaptoethanol. Cells were incubated alone and together with HBsAg 4 μ g/well (c) or 1 μ g/well (d) for 96 h and then pulsed with 1 μ Ci 3 H-thymidine overnight.

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Table 13 - Humoral response of BALB/c mice immunized i.m. with HBsAg or alum-adsorbed HBsAg, alone and combined with free/liposomal ISS-ODN

Vaccine (n=5)	IgG1 titer		IgG2a titer		IgG2a/IgG1 ratio		Anti-HBs (mIU/ml)	
	21 d	35 d	21 d	35 d	21 d	35 d	21 d	35 d
1. None	<10	<10	<10	<10	-	-	<10	<10
2. HBsAg 0.2 µg alone	100	1,500	<10	350	<0.1	0.23	15	823
3. HBsAg + ISS-ODN 10 µg	60	4,000	15	2500	0.25	0.62	18	1,183
4. HBsAg + lip ISS-ODN 10 µg	450	50,000	150	20,000	0.33	0.4	235	6,402
5. HBsAg + lip M-ODN 10 µg	500	20,000	<10	650	<0.02	0.03	25	2,286
6. HBsAg + empty liposomes	550	10,000	<10	250	<0.02	0.02	90	2,868
7. Lip ISS-ODN 10 µg alone	<10	<10	<10	<10	-	-	<10	<10
8. Alum-HBsAg alone	300	6,500	<10	30	<0.03	0.005	61	3,510
9. Alum-HBsAg + ISS-ODN	80	6,500	45	7,000	0.56	1.08	89	4,653
10. Alum-HBsAg + lip ISS-ODN	300	25,000	300	35,000	1.0	1.4	99	8,693

Table 14-The cellular response of BALB/c mice immunized i.m. with HBsAg or alum-adsorbed HBsAg, alone and combined with free/liposomal ISS-ODN

Vaccine	IFN γ (pg/ml) ^a	IL-4 (pg/ml) ^a	% Specific cytotoxicity E/T=50/1	Proliferation (SI) ^b
1. None	34 (0)	0 (0)	0	1.0 ^c /1.0 ^d
2. HBsAg 0.2 μ g alone	60 (0)	0 (0)	0	2.2/1.1
3. HBsAg + ISS-ODN 10 μ g	260 (0)	0 (0)	14	1.7/1.1
4. HBsAg + lip ISS-ODN 10 μ g	1300 (0)	15 (0)	35	1.8/1.4
5. HBsAg + lip M-ODN 10 μ g	11 (0)	28 (0)	0	2.5/1.9
6. HBsAg + empty liposomes	62 (0)	0 (0)	2	2.2/1.7
7. Lip ISS-ODN 10 μ g alone	11 (0)	0 (0)	0	1.1/1.1
8. Alum- HBsAg alone	180 (0)	20 (0)	5	3.5/2.6
9. Alum- HBsAg + ISS-ODN	420 (0)	12 (0)	24	2.0/1.9
10. Alum- HBsAg + lip ISS-ODN	1400 (0)	16 (0)	46	3.3/3.0

^a 0 = <10 pg/ml of either cytokine.

^b Stimulation index (SI) = mean cpm cells + antigen/mean cpm cells w/o antigen (^c 4 μ g/well, ^d 1 μ g/well).

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As can be seen in Table 13, free ISS-ODN administered together with HBsAg (group 3) was ineffective for the primary response (day 21) and moderately effective for the secondary response (day 35), increasing the levels of antigen-specific IgG1, IgG2a and anti-HBsAg antibodies 2.7, 7.4 and 1.4-fold, respectively, compared with antigen alone (group 2). In comparison, lip-ISS-ODN (group 4) was very effective for both the primary and the secondary response, being 5.4-13 times more efficient than free ISS-ODN for the various antibodies tested at the two time points. Interestingly, the combination of antigen and empty liposomes or lip M-ODN (groups 5, 6) induced high levels of IgG1 (but not IgG2a) and anti-HBs antibodies directing the response to a Th2-type (IgG2a/IgG1 ratio ≤ 0.03). Thus, the elevation of IgG1 by lip M-ODN can partly be attributed to the stimulatory action of the liposomes themselves. In contrast, free ISS-ODN and lip ISS-ODN elicited a mixed Th1-Th2 response (ratio, 0.25-0.62).

Vaccination with alum-adsorbed antigen (group 8) induced, as expected, high levels of both IgG1 and anti-HBs antibodies and a strong Th2 response (IgG2a/IgG1 ratio <0.03). When combined with free or lip ISS-ODN (groups 9, 10), again a mixed Th1-Th2 response (ratio, 0.56-1.4) was noted. Liposomal ISS-ODN was also 2-7 more effective than free ISS-ODN in combination with alum-HBsAg for the various antibodies tested.

Collectively, these results are in accordance with the data obtained in the influenza model described above (Tables 2–12), demonstrating the superiority of lip ISS-ODN over free ISS-ODN for enhancing the humoral response. However, whereas a Th1-dominant response was induced by lip ISS-ODN in the influenza model, when injected i.m., a mixed Th1-Th2 response is generated in the hepatitis B model.

The cellular responses were tested at 8 weeks, 5 weeks after the second vaccine dose (Table 14). Splenocytes were incubated alone and together with the antigen, and IFN γ , IL-4, cytotoxicity and proliferative response were measured. For the cytotoxic response, cultures were also carried out with 30 IU/ml of recombinant human IL-2 (Chiron, USA). IFN γ was produced only in antigen-stimulated cultures. Vaccination with HBsAg administered together with free ISS-ODN increased IFN γ production 4.7-

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fold compared with antigen alone; lip ISS-ODN induced 5 and 22 times more IFN γ than free ISS-ODN (groups 3, 4) and antigen alone (group 2), respectively. Lip M-ODN reduced IFN γ production and empty liposomes had no effect. Higher levels of IFN γ were generated following vaccination with alum-HBsAg, with and without
5 free/lip ISS-ODN (groups 8-10), than with HBsAg without alum. Lip ISS-ODN was 3.3 times more efficacious than free ISS-ODN. IL-4 levels were low (0-28 pg/ml) in all the vaccinated mice.

Significant specific cytotoxicity against HBsAg transfected P815 target cells was observed only in the groups immunized with HBsAg + free/lip ISS-ODN (groups
10 3, 4, 9, 10). The cytotoxicity obtained with lip ISS-ODN was 2-5-fold (depending on the formulation and the effector/target cell ratio) higher than that induced with free ISS-ODN. Cultures containing IL-2 showed a slightly higher cytotoxic activity with a similar pattern. In contrast with the stimulatory effects of free/lip ISS-ODN on IFN γ production and cytotoxicity, there was no effect of either formulation on the
15 proliferative response, which was low (stimulation index, 1.1-3.5) in all the groups. It is possible that the high level of IFN γ produced in the cultures blocked responder cell proliferation, or that the assay conditions were not optimal.

In conclusion, Table 14 shows that lip ISS-ODN was up to 5 times more potent than free ISS-ODN as an adjuvant with HBsAg vaccine with regard to IFN γ
20 production and cytotoxic activity. These finding complement the humoral response data (Table 13), indicating that lip ISS-ODN is a powerful Th1-dominant adjuvant at a low dose (10 μ g). In view of the ability of lip ISS-ODN to markedly boost both humoral and cellular responses to HBsAg, a combined vaccine consisting of the currently used HBV vaccine (alum- HBsAg) and lip ISS-ODN may be valuable not
25 only as a prophylactic vaccine, but as a therapeutic vaccine in chronic HBV carriers with active hepatitis B as well.

Example 3 *Enhancement of the systemic humoral response by liposomal ISS-ODN (Lip ISS-ODN) administered together with Tuberculosis vaccines*

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In a preliminary experiment, lip ISS-ODN 1018 was tested in mice as an adjuvant for a new recombinant *M. tuberculosis* vaccine. A combined vaccine composed of *M. tuberculosis* recombinant proteins (ESAT-6, L7/L12 and 85 B) mixed with Ribi adjuvant (Sigma, USA) was administered s.c. twice to female BALB/c mice (6-weeks old), alone and together with lip ISS-ODN (10 µg/dose, prepared as described above). Free ISS-ODN was not tested in this experiment. Antigen-specific IgG1 and IgG2a were quantified by ELISA 3 weeks after the second vaccine dose. The results are shown in the following Table 15.

10 **Table 15**-Serum IgG isotypes of BALB/c mice immunized s.c. with recombinant *M. tuberculosis* proteins, with and without lip ISS-ODN

Vaccine (n=3)	Mean optical density with: ^a					
	ESAT-6		L7/L12		85B	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
Ag + Ribi	0.178	0.104	0.285	0.116	0.210	0.190
	(0.6)		(0.4)		(0.9)	
Ag + Ribi + lip ISS-ODN	0.363	0.498	0.411	0.660	0.358	0.633
	(1.4)		(1.6)		(1.8)	
Fold increase	2.0	4.8	1.4	5.7	1.7	3.3

^a Tested by ELISA against each antigen individually on sera diluted 1/4000; background OD reading was 0.05 (in parentheses, the IgG2a/IgG1 ratio)

15 As shown in Table 15, administration of lip ISS-ODN together with the tuberculosis vaccine increased both IgG1 and IgG2a against the 3 proteins, particularly IgG2a (1.4-2-fold vs. 3.3-5.7-fold, respectively, for IgG1 and IgG2a). This resulted in an increased IgG2a/IgG1 ratio from 0.6, 0.4 and 0.9 without lip ISS-ODN to 1.4, 1.6 and 1.8 with lip ISS-ODN, respectively, indicating a shift to a Th1 response.

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In an additional experiment, Lip ISS-ODN 1018 was compared with free ISS-ODN as an adjuvant for Tuberculosis vaccine comprising a different mixture of antigens. According to this specific experiment, In general, BALB/c mice (females, 2-months-old, n=5/group) were vaccinated subcutaneously on days 0 and 21 using a mixture of
 5 3 different antigens (5 µg each) derived from *M. tuberculosis*. ODN 1018-10 µg/dose. The results presented in Table 16 show that lip ISS-ODN was 2–2.5-fold more potent for two of the three antigens tested.

These results suggest that lip ISS-ODN may prove beneficial as a parenteral Th1 adjuvant for future tuberculosis vaccines.

10

Table 16-Soluble and liposomal ISS-ODN (ODN 1018) as adjuvants for tuberculosis vaccine in mice (exp. 2)

Vaccine	Antigen	IgG1 titer (x10 ³) ^a	IgG2a titer (x10 ³)	IgG2a/IgG1 ratio
Antigen alone	Sod	13	7	0.54
Antigen + ODN 1018		12	11	0.91
Antigen + lip ODN 1018		22	26	1.18
Antigen alone	85B	11	8	0.72
Antigen + ODN 1018		10.5	8	0.76
Antigen + lip ODN 1018		13	16.5	1.27
Antigen alone	CFP21	21	8.5	0.4
Antigen + ODN 1018		110	23	0.21
Antigen + lip ODN 1018		105	22	0.21

^a Pooled sera were tested by ELISA 3 weeks after the second vaccine dose. ELISA titer was determined as the highest serum dilution yielding OD=0.2.

Example 4 *Lip ISS-ODN 1018 as an adjuvant for cancer vaccine*

A weakly immunogenic murine mammary carcinoma (4T1) was used in 2 experiments. Female, 2-months-old (BALB/c x C57BL6) F1 mice were vaccinated 3 times, intradermally, on days 0, 10 and 20 with either irradiated tumor cells only, tumor cells admixed with 10 µg per dose of free or liposomal ISS-ODN 1018, or with empty liposomes. Tumor challenge was injected subcutaneously 10 days after the last vaccination (injection of 5×10^4 live tumor cells) and mice were inspected for tumor incidence and size 4 weeks later. As shown in Table 17, only groups co-vaccinated with ISS-ODN were partially protected, with 33% and 25% protection achieved with Lip ISS-ODN and free ISS-ODN, respectively. In a similar experiment the protection rate was 37% (3/8) vs. 25% (2/8), respectively. Thus, under the experimental conditions used, Lip ISS-ODN was slightly more effective than free ISS-ODN. Optimization of the vaccination procedure is required to further increase the protection rate.

15

Table 17-Soluble and liposomal ISS-ODN (ODN 1018) as adjuvants for cancer vaccine in mice

Vaccine^a	No. of tumor-bearing mice/total^b on day 28	Mean tumor size (cm³)^b on day 28
1. None	8/8	1.7
2. Tumor alone	8/8	0.6
3. Tumor + soluble ODN 1018	6/8	0.44
4. Tumor + liposomal ODN 1018	6/9	0.29
5. Tumor + empty liposomes	8/8	0.78

^a vaccination with 10^7 irradiated (20,000R) 4T1 cells at 4 sites either alone or together with soluble or liposomal ODN 1018 or with empty liposomes as described.

20 ^b Live tumor cells (5×10^4) were injected intradermally 10 days after the third vaccine dose.

Example 5 *Activation of resistance to Leishmaniasis by liposomal ISS-ODN (Lip ISS-ODN) administered after infection*

Resistance to leishmaniasis requires activation of a Th1-type response.
5 Therefore, free and liposomal (lip) ISS-ODN 1018 were tested as post-infection treatment for leishmaniasis in BALB/c mice. In particular, animals (8-9/group) were injected subcutaneously (s.c.) in the tail base with 250,000 *Leishmania major* promastigotes. Saline, free ISS-ODN, lip ISS-ODN or lip M-ODN (lacking the CpG motif) were administered intramuscularly (i.m., 0.1 ml) at 20 µg/dose on days 1, 7, 14
10 (cycle 1) and 72 and 82 (cycle 2) post infection (lip ISS-ODN or lip M-ODN comprising DMPC/DMPG at mole ratio of 9:1 and lipid:ODN ratio (w/w) of 100:1, prepared as described above). Subcutaneous lesions were measured weekly by caliper.

As shown in Table 18 and in Fig. 1, free ISS-ODN demonstrated a moderate protective effect. Animals treated with lip ISS-ODN showed a slower tempo of disease
15 development, and the lesion size after each treatment cycle was about half of that seen in the other groups, including mice treated with free ISS-ODN.

In two additional experiments mice were infected as above, then treated with a subcurative dose of anti-leishmanial drugs (Amphotericin B (Fig. 2) and Amphotericin B derivative (Fig. 3)), alone and combined with lip ISS-ODN. Other
20 groups were treated with either lip ISS-ODN or lip M-ODN (lacking the CpG motif) only. As shown in Figs. 2 and 3, a synergistic therapeutic effect was achieved in the group co-treated with the drug and the lip ISS-ODN. Since the therapeutic effect is transient and partial, repeated treatment over a long period of time, or a combination of higher doses of lip ISS-ODN with higher doses of anti-leishmanial drugs may be
25 necessary to achieve a complete cure.

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Table 18-Post-infection treatment of leishmaniasis in BALB/c mice with free/liposomal ISS-ODN (cycle 1)

Treatment ^a	Lesion size (mm ² , mean \pm SD) on week: ^b	
	4	8
Saline	1.44 \pm 1.22 (1/8)	11.67 \pm 7.9 (0/8)
ISS-ODN 20 μ g	0.74 \pm 1.08 (4/9)	8.3 \pm 6.8 (1/9)
Liposomal ISS-ODN 20 μ g	0.46 \pm 1.04 (6/9)	3.8 \pm 3.5 (1/9)
Liposomal M-ODN 20 μ g	1.35 \pm 1.67 (2/8)	13.2 \pm 10.1 (1/8)

^b Two diameters were measured for calculation of the lesion area. In parentheses, number of mice without lesions/total number of mice.

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CLAIMS:

1. A method for loading immunostimulatory oligodeoxynucleotides (ISS-ODNs) in liposomes comprising:
 - (a) solubilizing at least one liposome-forming lipid in a solvent and drying
5 same to effect a dry liposome-forming lipid or dry mixture of lipids;
 - (b) providing an aqueous solution of ISS-ODN;
 - (c) hydrating the dry liposome-forming lipid or mixture of lipids with said ISS-ODN solution to achieve loading of said ISS-ODN in liposomes.
2. A method for loading immunostimulatory oligodeoxynucleotides (ISS-ODNs)
10 in liposomes comprising:
 - (a) solubilizing at least one liposome-forming lipid in a solvent and freeze-drying same to effect a dry lipid or dry mixture of lipids;
 - (b) providing an aqueous solution of ISS-ODN;
 - (c) hydrating the freeze-dry lipid or mixture of lipids with said ISS-ODN
15 solution to achieve loading of said ISS-ODN in liposomes.
3. The method of Claim 1 or 2, wherein said liposome-forming lipid comprises phospholipid, lipopolymers, sphingolipids a combination thereof and a combination thereof with sterols.
4. The method of Claim 3, wherein said phospholipid is selected from saturated or
20 unsaturated phospholipids, hydrogenated, partially hydrogenated or non-hydrogenated phospholipids, fully or semi synthetic phospholipids.
5. The method of Claim 3, wherein said hydrogenated, partially hydrogenated or non-hydrogenated phospholipids are derived of natural sources, said natural source is selected from egg yolk, milk, rice or soybeans.
- 25 6. The method of Claim 4, wherein said fully synthetic or semi-synthetic phospholipids are selected from dimyristoyl phosphatidylcholine (DMPC), dimyristoyl

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phosphatidylglycerols (DMPG), phosphatidylglycerols, phosphatidylinositols, phosphatidylserines, sphingomyelins, or mixture thereof.

7. The method of Claim 6, wherein the liposome-forming lipids comprises a mixture of DMPC and DMPG.
- 5 8. The method of Claim 7, wherein said mixture of DMPC and DMPG is at a molar ratio of between 1:20 and 20:1
9. The method of Claim 3, wherein said lipopolymer is a PEGylated lipid.
10. The method of Claim 3, wherein said sphingolipids are sphingomyelins (SPM) selected from egg-derived SPM, milk-derived SPM, N-palmitoyl-SPM,
10 N-stearoyl-SPM, N-oleoyl-SPM (C18:1), N-nervacyl C (C24:1) SPM, N-lignoceryl SPM (C24:0), or a mixture thereof.
11. The method of Claim 1 or 2, wherein said ISS-ODN is loaded into said liposome in combination with an antigen or mixture of antigens.
12. The Method of Claim 1 or 2, wherein said ISS-ODN is an endotoxin-free ISS-
15 ODN with a phosphorothioate (PS) or phosphodiester (PO) backbone.
13. The method of Claim 1 or 2, wherein said solvent is a polar, water miscible solvent.
14. The method of Claim 13, wherein said solvent is tertiary-butanol.
15. The method of Claim 1 and 2 wherein the solvent is an apolar solvent.
- 20 16. The method of Claim 15, wherein said apolar solvent is cyclohexane.
17. The method of Claim 1, wherein said drying includes spray drying.
18. The method of Claim 1 or 2, wherein said solution of ISS-ODN is a solution thereof in sterile pure water, a physiologically acceptable aqueous solution selected from 0.9% NaCl, buffered Saline, 5% dextrose, buffered dextrose, 10% sucrose and
25 buffered sucrose or cryoprotectant or a mixture of same.

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19. A combination of two pharmaceutical compositions including a first pharmaceutical composition comprising dry liposome-forming lipids and a second pharmaceutical composition comprising ISS-ODN, the combination is for use in the preparation of a pharmaceutical formulation comprising liposomal ISS-ODN.
- 5 20. The combination of Claim 19, being in the form of a package.
21. The combination of Claim 19 or 20, comprising instructions for use of said first pharmaceutical composition and of said second pharmaceutical composition for the preparation of said pharmaceutical composition, the instructions comprising hydrating the dry liposome forming lipid or mixture of lipids of the first composition with an
10 aqueous solution of the second composition containing ISS-ODN; and further comprising instructions prescribing administration of said pharmaceutical formulation to a subject in need thereof.
22. The combination of Claim 21, comprising a physiologically acceptable aqueous medium, sterile water and/or cryoprotectant, for forming a solution of ISS-ODN.
- 15 23. The combination of Claim 19, wherein the liposome-forming lipid comprises phospholipid, lipopolymers, sphingolipids and combination of the same with sterols
24. The combination of Claim 23, wherein said liposome-forming lipid comprises saturated or unsaturated phospholipids, hydrogenated, partially hydrogenated or non-hydrogenated phospholipids, fully or semi synthetic phospholipids.
- 20 25. The combination of Claim 24, wherein said hydrogenated, partially hydrogenated, or non-hydrogenated phospholipids are derived from a natural source; said natural source is selected from egg yolk, milk, rice or soybeans.
26. The combination of Claim 24, wherein said fully synthetic or partially synthetic phospholipids are selected from dimyristoyl phosphatidylcholine (DMPC), dimyristoyl
25 phosphatidylglycerol (DMPG), phosphatidylglycerol, phosphatidyl-inositol, phosphatidylserine, or mixture thereof.
27. The combination of Claim 26, wherein said liposome forming lipid comprises a mixture of DMPC and DMPG.

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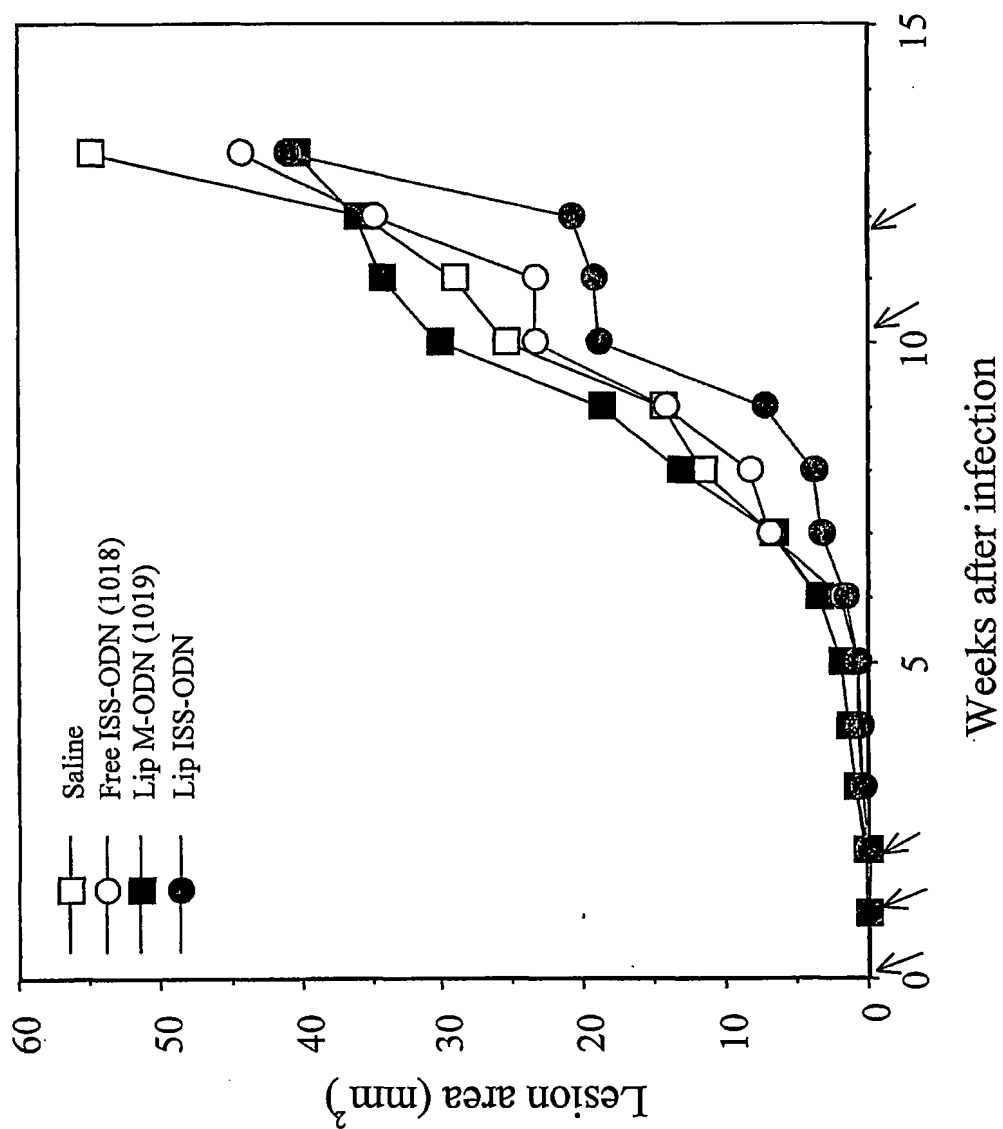
28. The combination of Claim 27, wherein said mixture DMPC and DMPG is at a molar ratio of between 1:20 and 20:1.
29. The combination of Claim 23, wherein said lipopolymer is PEGylated lipids.
30. The combination of Claim 23, wherein said sphingolipids are sphingomyelins
5 (SPM) selected from egg-derived SPM, milk-derived SPM, N-palmitoyl-SPM, N-stearoyl-SPM, N-oleoyl-SPM (C18:1), nervonyl C (C24:1) SPM, N-lignoceryl SPM (C24:0), or a mixture thereof.
31. The combination of Claim 19, wherein said ISS-ODN is an endotoxin-free ISS-ODN with a phosphorothioate (PS) or phosphodiester (PO) backbone.
- 10 32. The combination of Claim 19 or 31, wherein said second composition comprises, in combination with said ISS-ODN, an antigen or a mixture of antigens.
33. The combination of Claim 22 or 31, wherein said physiologically acceptable aqueous medium is sterile water or a solution selected from the group consisting of 0.9% NaCl, buffered Saline, 5% dextrose, buffered dextrose, 10% sucrose, buffered
15 sucrose and mixtures of the same.
34. A pharmaceutical formulation comprising as active ingredient a therapeutically effective amount of liposomes loaded with an ISS-ODN and a pharmaceutically acceptable additive, the liposomal ISS-ODN being prepared by the method of any one of Claims 1 to 17.
- 20 35. The pharmaceutical composition of Claim 34, wherein said effective amount is a dosage of up to 2,000 mg of loaded liposomal vesicles, measured by phospholipid, per kg body weight.
36. The pharmaceutical formulation of Claim 34 comprising, in combination with said ISS-ODN, an antigen or a mixture of antigens, said antigen(s) is in a free form or
25 loaded into a liposome.
37. The pharmaceutical formulation of Claim 36, wherein said antigen is loaded together with the ISS-ODN in the same liposome or in separate liposomes.

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38. The pharmaceutical formulation of any one of Claims 34 to 37, for eliciting an immune response of an individual.
39. A method for the prevention or treatment of a disease comprising administering to a subject in need an effective amount of pharmaceutical formulation according to
5 any one of Claims 34 to 38.
40. A method for stimulating the immune response of an individual, the method comprising administration to said individual an amount of ISS-ODN-loaded liposome effective to stimulate said immune response, wherein said stimulation is to an extent greater than that obtained by administration to the individual free ISS-ODN, the ISS-
10 ODN-loaded liposome being prepared by the method of any one of Claims 1 to 19.
41. The method of Claim 40, wherein said amount is a dosage of up to 2,000 mg of loaded liposomal vesicles, measured by phospholipid, per kg body weight.
42. The method of Claim 40, comprising administration of said liposomal ISS-ODN in combination with at least one antigen.
- 15 43. The method of Claim 42, wherein said antigen is in a free form.
44. The method of Claim 42, wherein said antigen is encapsulated together with said ISS-ODN in the same liposomes.
45. The method of Claim 42, wherein said antigen is encapsulated in a liposome separate from said ISS-ODN.

1/3

Fig. 1



2/3

Fig. 2

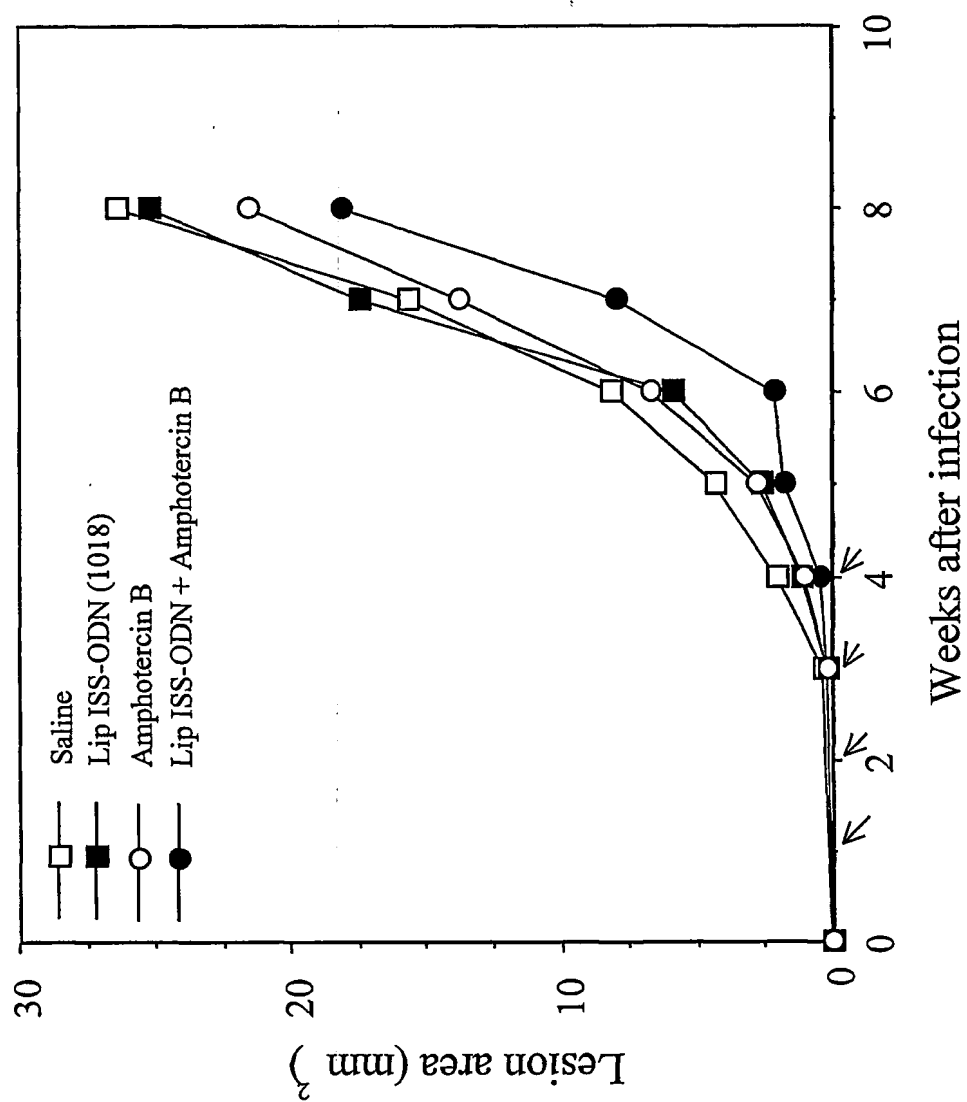
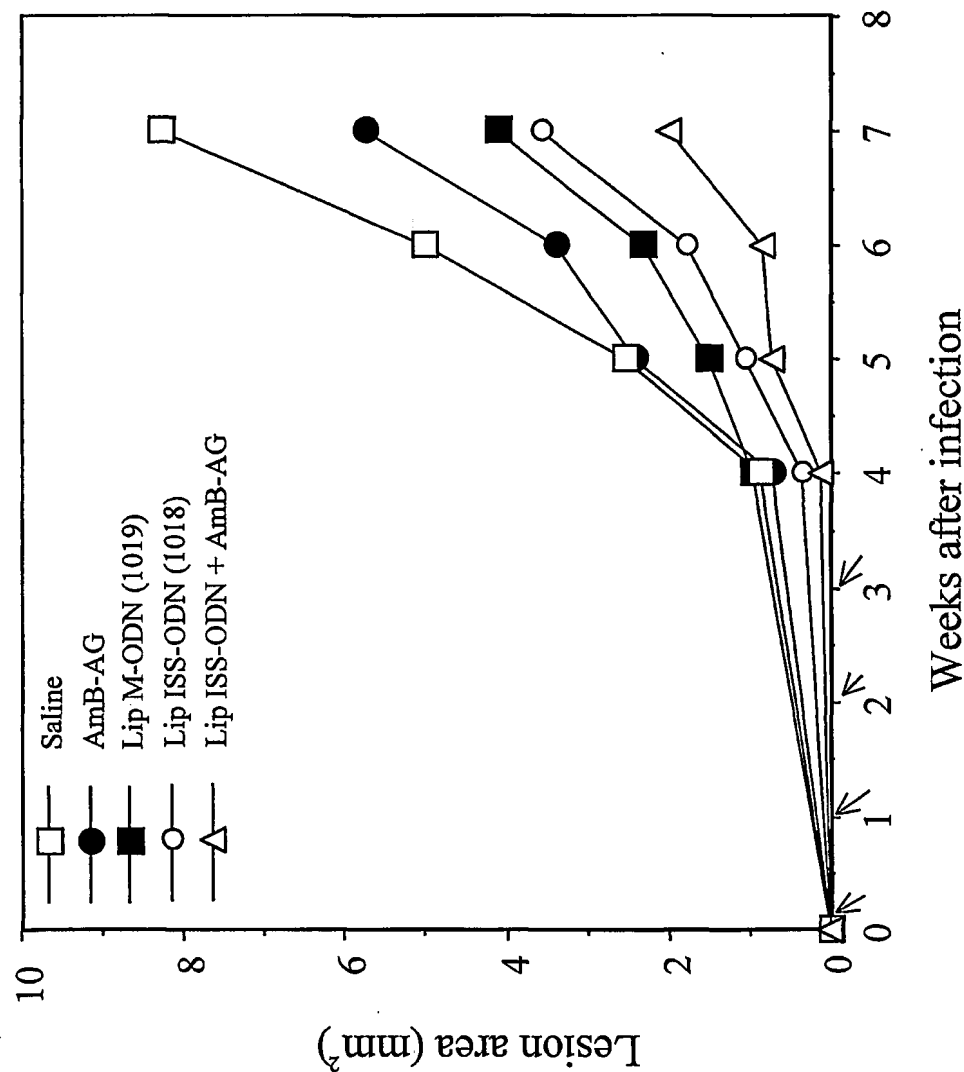


Fig. 3



SEQUENCE LISTING

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<120> A METHOD FOR PREPARATION OF VESICLES LOADED WITH IMMUNOSTIMULATORY OLIGODEOXYNUCLEOTIDES AND DIFFERENT USES THEREOF

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23

Enhanced Dendritic Cell Maturation by TNF- α or Cytidine-Phosphate-Guanosine DNA Drives T Cell Activation In Vitro and Therapeutic Anti-Tumor Immune Responses In Vivo¹

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Dendritic cells (DC) manipulated ex vivo can induce tumor immunity in experimental murine tumor models. To improve DC-based tumor vaccination, we studied whether DC maturation affects the T cell-activating potential in vitro and the induction of tumor immunity in vivo. Maturation of murine bone marrow-derived DC was induced by GM-CSF plus IL-4 alone or by further addition of TNF- α or a cytidine-phosphate-guanosine (CpG)-containing oligonucleotide (ODN-1826), which mimics the immunostimulatory effect of bacterial DNA. Flow cytometric analysis of costimulatory molecules and MHC class II showed that DC maturation was stimulated most by ODN-1826, whereas TNF- α had an intermediate effect. The extent of maturation correlated with the secretion of IL-12 and the induction of alloreactive T cell proliferation. In BALB/c mice, s.c. injection of colon carcinoma cells resulted in rapidly growing tumors. In this model, CpG-ODN-stimulated DC cocultured with irradiated tumor cells also induced prophylactic protection most effectively and were therapeutically effective when administered 3 days after tumor challenge. Thus, CpG-ODN-enhanced DC maturation may represent an efficient means to improve clinical tumor vaccination. *The Journal of Immunology*, 2000, 165: 6278–6286.

Dendritic cells (DC)⁵ are highly specialized APCs that are unique in their potency to induce T cell-dependent Ag-specific immune responses (1). After capturing and processing Ag in the periphery, they migrate to the T cell areas of lymph nodes or spleen where they sensitize naive T cells and direct their development into Ag-specific effector cells (2–4). Contact to “danger signals” like LPS, TNF- α , IL-1, or CD40 ligand expressed by activated T cells leads to DC maturation (5, 6) and IL-12 secretion (7–9). IL-12 plays a central role in the tumor-directed im-

mune response, stimulating NK, cytotoxic CD8⁺ T, and IFN- γ -producing CD4⁺ Th1 cells. Through its stimulatory activity on undifferentiated CD4⁺ precursors to develop into Th1 cells, IL-12 antagonizes the action of IL-4, which promotes Th2 development (10–12).

Synthetic oligodeoxynucleotides containing unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides (CpG-ODN) in specific sequence contexts mimic the immunostimulatory qualities of bacterial DNA (13). In vitro, they up-regulate the expression of costimulatory and Ag-presenting molecules and the secretion of IL-12 by monocytes and DC (7, 9, 14–16). In vivo, CpG-ODN act as an adjuvant, promoting Th1 immune responses (17, 18) that can enhance protection from a subsequent tumor challenge when coadministered with tumor Ag (19).

In murine tumor models, DC act as potent inducers of tumor immunity. Tumor development was induced by injection of established tumor cell lines of various tissue origins. Following interaction with tumor cells (20, 21) or selected tumor Ags (22–25), DC are effective as prophylactic tumor vaccines against subsequent tumor challenges. In a few cases, even therapeutic efficacy of DC-based tumor vaccines was reported, leading to rejection of established tumors and lung metastases. These vaccines were based on fusion (20) or coculture (21) of DC and tumor cells, on tumor peptide- (26) or tumor lysate-pulsed DC (27). In the two studies reporting complete remission of established tumors, DC were generated in the presence of GM-CSF (20) or of GM-CSF plus IL-4 (21) without additional DC-activating agents. Both research teams worked with the C57BL/6 mouse strain, which is prone to raise proinflammatory, Th1-mediated immune responses (28) and injected low to moderate aggressively growing tumor cells. However, in man, aggressive tumor growth is often met by a severely compromised immune response. First clinical trials applying DC

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² C.B. and J.S. contributed equivalently to this work.

³ This work contains parts of the doctoral thesis of J.S. and that of A.S. at the Ludwig-Maximilians-University of Munich.

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⁵ Abbreviations used in this paper: DC, dendritic cell(s); CpG, cytidine-phosphate-guanosine; CpG-ODN, synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides; MFI, mean fluorescence intensity; MHC II, MHC class II; MPL, monophosphoryl lipid A.

(29–31) have demonstrated therapeutic benefit (partial or complete regressions) that was, however, limited to a small proportion of patients. As inflammatory stimuli induce maturation of DC (5, 6), characterized by increased expression of MHC and costimulatory molecules, we reasoned that they may improve the therapeutic potential of DC-based vaccines.

Toward this goal, we studied potential improvements and limits of DC tumor vaccines. We tested whether tumor vaccination was effective against aggressively growing, syngeneic colon carcinoma cells in BALB/c mice, which have an impaired Th1 response (32). We analyzed how enhanced maturation of DC compared with basic stimulation by GM-CSF and IL-4 alone influences their potential to induce tumor immunity, both prophylactically and therapeutically. In addition, systemic administration of CpG-ODN as an adjuvant with or without tumor cells was investigated. The concomitant *in vitro* characterization of differentially stimulated DC allowed correlation of the surface expression of DC marker proteins, the IL-12 synthesis and the T cell activation potential with the anti-tumor efficacy observed in the mouse model.

Materials and Methods

Mice and cell lines

Female BALB/c mice, 6–8 wk old, were purchased from Harlan Winkelmann (Borchen, Germany). Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The murine, BALB/c-derived Colon-26 carcinoma line as well as the murine Renca cells (Cell Lines Service, Heidelberg, Germany) were maintained in DMEM supplemented with 10% FCS, 1% L-glutamine, and antibiotics.

Preparation of DC

DC were prepared as described (33) with minor modifications. Bone marrow leukocytes from mouse tibia and femur were depleted of T and B lymphocytes and granulocytes by incubation (30 min, 4°C) with rat anti-mouse CD4, CD8, Ly6G, and CD45R mAbs (Serotec, Oxford, U.K.) followed by incubation (15 min, 4°C) with goat anti-rat IgG conjugated to magnetic beads (Dynal, Oslo, Sweden) and separation in a magnetic field. Unbound cells were cultured in DC medium consisting of RPMI 1640 medium containing 10% FCS, 1% L-glutamine, and antibiotics supplemented with rGM-CSF (200 U/ml; Roche, Mannheim, Germany) and IL-4 (PeproTech, London, U.K.). After 7 days, loosely adherent cells were harvested, and expression of MHC class II (MHC II), CD40, CD54, CD80, and CD86 was quantified by flow cytometry. In some experiments at day 7 or 9, DC were additionally stimulated with 5–80 ng/ml murine TNF- α (R&D Systems, Minneapolis, MN) or with 6 μ g/ml phosphorothioate-modified oligonucleotide ODN-1826 described by Yi (34) 5'-TCC ATG ACG TTC CTG ACG TT-3' (provided by Coley Pharmaceutical Group, Wellesley, MA). To assess the specificity of ODN-1826, stimulation of DC with 6 μ g/ml phosphorothioate-modified oligonucleotide ODN-1982 5'-TCC AGG ACT TCT CTC AGG TT-3' served as control.

Flow cytometry

Cells were incubated for 30 min at 4°C with 5 μ g/2 $\times 10^5$ cells rat anti-mouse MHC II, CD40, CD54, CD80, and CD86 mAbs (PharMingen, San Diego, CA and Serotec, Oxford, U.K.). After washing with PBS containing 2% horse serum, FITC-conjugated mouse anti-rat IgG (Dianova, Hamburg, Germany) was added for 20 min at 4°C. After washing in PBS, samples were analyzed using an EPICS Profile II flow cytometer (Coulter, Miami, FL).

Quantification of IL-12

The concentration of IL-12p40-subunit in culture supernatants was determined by ELISA according to the manufacturer's protocol (R&D Systems).

Allogeneic T cell proliferation

T cells were isolated by passing spleen from C57BL/6 mice through a 70- μ m cell strainer (Falcon, Heidelberg, Germany) followed by lysis of erythrocytes (Ortho Diagnostic Systems, Neckargemünd, Germany) and magnetic bead conjugate-mediated depletion of B lymphocytes and granulocytes with rat anti-mouse CD45R and Ly6G mAbs (Serotec). T cells were 60–80% pure as determined by flow cytometry.

Bone marrow-derived day 7 DC were exposed to ionizing radiation (50 Gy). Varying numbers of irradiated DC (600, 3,000, or 15,000 cells) were cocultured with 1.5×10^5 allogeneic T cells in 96-well culture plates for 48 h. [3 H]thymidine (1 μ Ci; Amersham Buchler, Braunschweig, Germany) per well was added for 24 h, cells were harvested, and [3 H]thymidine incorporation was counted in a Betaplate scintillation counter (Wallac, Turku, Finland).

DC tumor cell coculture and immunization

DC (5×10^6) were cocultured with 1×10^6 irradiated tumor cells (100 Gy) for 4 days in culture medium supplemented with GM-CSF (200 U/ml) and IL-4 (20 ng/ml). For additional DC stimulation, CpG 1826 (6 μ g/ml) or TNF- α (20 ng/ml) was added for the last 2 days. At day 11, loosely adherent cells were harvested, washed 3 times, and resuspended in HBSS (Life Technologies, Karlsruhe, Germany). For prophylactic immunization, 1×10^6 cells were injected s.c. into the right lower flank (200 μ l). Seven days later, mice were challenged by ipsilateral injection of Colon-26 cells (5×10^5 in 200 μ l HBSS). To assess tumor specificity, mice were challenged by injection of Renca cells (5×10^5 in 200 μ l HBSS) 7 days after immunization with 1×10^6 DC cocultured with irradiated Colon-26 cells. For therapeutic immunization, 1×10^6 coculture cells were injected at the indicated days after tumor challenge (1×10^5 or 5×10^5 Colon-26 cells in 200 μ l HBSS). For vaccination with oligonucleotide as adjuvant, 50 μ g CpG-ODN 1826 was injected s.c. alone or in combination with 5×10^5 irradiated tumor cells (100 Gy) in 200 μ l HBSS. Seven days later, mice were challenged ipsilaterally with 5×10^5 tumor cells. According to the guidelines for the proper use of laboratory animals, mice were killed when severely suffering (e.g., shivering and showing decreased mobility). The decision was made by an investigator blinded toward the treatment allocation.

Statistics

Student's *t* test was applied to reveal significant differences in tumor protection by differently stimulated DC. We compared the mean tumor size of controls and all treated animals (tumor-free and tumor-positive animals). In addition, we compared the mean of controls with the mean of only tumor-positive animals (tumor-free animals in treatment group excluded). A value of *p* < 0.05 was accepted as the level of significance.

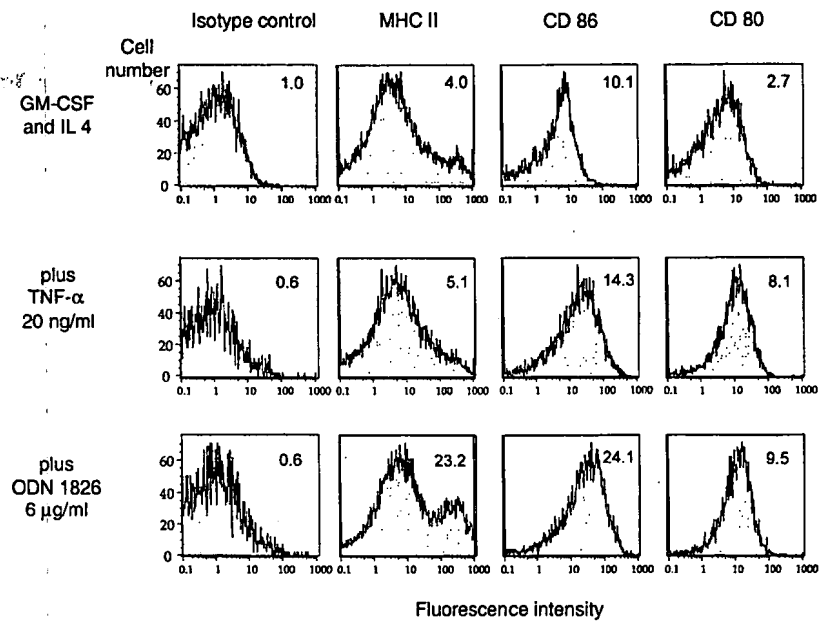
Results

ODN-1826 induces higher DC maturation than TNF- α

The pronounced expression of MHC and costimulatory molecules contributes to the ability of DC to potentially activate T cells. As surface expression of these proteins increases during differentiation, induction of maturation may enhance the immunostimulatory capacity of DC. Fig. 1 depicts the effect of different culture conditions on the maturation of DC. Basal stimulation with GM-CSF and IL-4 alone (Fig. 1, *upper panel*) generates DC that express moderate levels of CD80 (mean fluorescence intensity (MFI) 2.8), CD86 (MFI 10.1), and MHC II (MFI 3.9). The cells also expressed CD40 and CD54 and were identified morphologically as DC by phase contrast microscopy of May-Grünwald-stained cytopins and by raster electron microscopy (data not shown). TNF- α enhanced the surface density of CD80 (MFI 8.2), CD86 (MFI 14.3), and MHC II (MFI 5.1, Fig. 1, *middle panel*). An even stronger increase of the surface expression of costimulatory and Ag-presenting molecules was induced by ODN-1826 (Fig. 1, *lower panel*). On average, CD80 was increased 4-fold (MFI 9.5), CD86 2-fold (MFI 24.1), and MHC II 6-fold (MFI 23.2) compared with DC stimulated with GM-CSF and IL-4 alone.

We noticed a dose-dependent effect of ODN-1826 with 500, 2000, and 6000 ng/ml inducing a CD80 expression of 80, 83, or 86% and a CD86 expression of 84, 87, or 90%, respectively. Analyzing the expression of MHC II, no significant dose-related differences could be observed (data not shown). Additional stimulation of ODN-1826-activated DC with 100 ng/ml LPS did not induce any further increase of the MFI of MHC II, CD86, and CD80 (data not shown), indicating maximal stimulation of ODN-1826-stimulated DC (data not shown). ODN-1826-stimulated DC placed in fresh medium maintained their dendritic, nonadherent

FIGURE 1. Effect of differential DC stimulation on expression of MHC II and costimulatory molecules. Murine bone marrow-derived precursor cells were cultured in the presence of 200 U/ml GM-CSF and 20 ng/ml IL-4 for 7 days alone (*upper panel*) or additionally stimulated with 20 ng/ml TNF- α (*middle panel*) or with 6 μ g/ml ODN-1826 (*lower panel*). Surface density of MHC II, CD86, and CD80 was analyzed by flow cytometry. One representative experiment of three is shown.



phenotype for at least 1 wk without GM-CSF, IL-4, or additional stimulation.

Effect of IL-4, TNF- α , and ODN-1826 on DC IL-12 synthesis

DC secrete IL-12 (7–9), a key inducer of proinflammatory Th1 responses. We investigated to what extent stimulation of DC influenced IL-12 secretion. As IL-4 has antagonistic effects, promoting anti-inflammatory Th2 cytokine profiles, we tested whether IL-4 (which is frequently added to DC cultures to increase DC survival and differentiation) inhibits IL-12 synthesis by DC. DC were cultured in the presence of GM-CSF and different concentrations of IL-4 (0, 20, and 80 ng/ml) for 7 days. IL-4 had no inhibitory effect on baseline IL-12 synthesis (Fig. 2a). However, IL-4 addition increased DC yield. TNF- α increased the IL-12 secretion induced by GM-CSF and IL-4 alone up to 4-fold (Fig. 2b). ODN-1826 induced an extremely high IL-12 production, 19-fold higher than after basic stimulation with GM-CSF and IL-4 alone. The extent of IL-12 secretion induced by TNF- α and ODN-1826 correlated with enhancement of surface expression of CD80, CD86, and MHC II (Fig. 1).

Effect of DC maturation on T cell activation in vitro

A major objective of tumor vaccination is enhancement of tumor-specific T cell responses. To determine the effect of DC maturation on T cell activation in vitro, we compared DC generated with GM-CSF and IL-4 alone to TNF- α - or ODN-1826-stimulated DC in their capacity to induce allogeneic T cell proliferation (Fig. 3). Even at a relatively high DC number (DC-T cell ratio, 1:10), DC grown in the presence of GM-CSF and IL-4 alone marginally activated T cells. T cell proliferation induced by spleen cells was even lower (data not shown). In contrast, TNF- α - or ODN-1826-activated DC induced high T cell proliferation and there was a 1.3-fold increase between these latter preparations. However, at lower DC numbers (DC-T cell ratios, 1:50 and 1:250) we observed marked differences in the capacity of the respective DC to induce T cell proliferation. At a DC-T cell ratio of 1:50, TNF- α -activated DC induced a 2-fold (5 ng/ml TNF- α), a 4-fold (20 ng/ml TNF- α), and a 9-fold (80 ng/ml TNF- α) higher T cell proliferation compared with GM-CSF- and IL-4-stimulated DC, but were only marginally more effective at low DC numbers (DC-T cell ratio, 1:250).

In contrast, ODN-1826-activated DC were potent T cell stimulators also at low numbers, inducing a 15-fold (1:50) and 12-fold (1:250) higher T cell proliferation than GM-CSF- and IL-4-stimulated DC. Thus, at low numbers, only highly matured DC were able to activate T cells. The effect of TNF- α - or ODN-1826-induced DC activation on T cell proliferation again correlated with the respective effects observed on expression of DC marker proteins and on IL-12 secretion.

To determine the specificity of the immunostimulatory effect of ODN-1826 on murine DC, we repeated our in vitro experiments using the inactive control oligo ODN-1982. In all three experiments, ODN-1982-stimulated DC did not show an increased surface expression of MHC II, CD80, or CD86 compared with DC stimulated with GM-CSF and IL-4 alone. In contrast to ODN-1826-stimulated DC, no enhanced IL-12 secretion could be induced by stimulation with ODN-1982 compared with DC stimulated in GM-CSF and IL-4 alone. This correlates with the result of the T cell proliferation assays in vitro, where ODN-1982-stimulated DC showed an activation of T cells even below the level induced by DC stimulated with GM-CSF and IL-4 alone (data not shown).

DC maturation and physical contact to tumor cells in vitro improves protective tumor immunity in vivo

We evaluated whether enhanced maturation of DC and coculture with tumor cells improves the induction of protective tumor immunity. DC stimulated with GM-CSF and IL-4 for 7 days were cocultured with irradiated tumor cells at a ratio of 5:1 for 4 days. For the last 48 h of coculture, DC were activated with either TNF- α or ODN-1826. Mice were vaccinated s.c. with 1×10^6 cocultured cells and, 7 days later, mice were challenged with 5×10^5 viable tumor cells. Three independent experiments ($n = 45$) were performed, and one representative result with five mice per group is shown in Fig. 4. Tumor growth in unvaccinated control mice was rapid, leading to death (by natural causes or by tumor burden-based euthanasia) by day 18, demonstrating the aggressive tumor formation by Colon-26 cells in BALB/c mice (Fig. 4, O). As a mean of all three experiments, 31% of mice immunized with DC that were cocultured with tumor cells in the presence of GM-CSF and IL-4 were completely protected against tumor formation (no

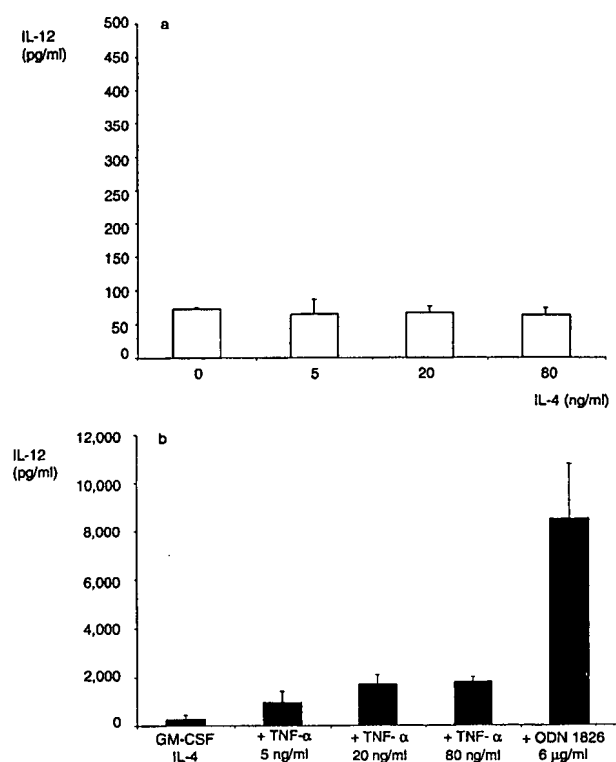


FIGURE 2. IL-12 secretion by DC. Bone marrow-derived precursor cells were cultured in the presence of 200 U/ml GM-CSF plus varying doses of IL-4 (*a*) or, in the presence of 200 U/ml GM-CSF, 20 ng/ml IL-4 and varying doses of TNF- α or 6 μ g/ml ODN-1826 (*b*). At day 7 of culture, supernatants were measured by ELISA. The means \pm SD of three experiments are shown.

development of tumors until day 21 and for at least 4 wk beyond). In the remaining 69% of mice, tumor growth was slightly delayed with survival up to day 21 (Fig. 4*a*, $p < 0.001$). Injection of 5×10^5 Colon-26 cells 7 days after vaccination with ODN-1826-stimulated DC that had not been exposed to tumor cells led to s.c. tumor formation in all mice ($n = 10$, data not shown). Vaccination with TNF- α -stimulated DC cocultured with tumor cells rendered half of the mice (50%) resistant to tumor challenge while delaying

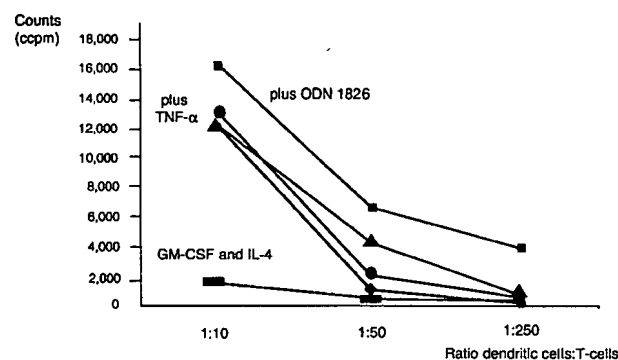


FIGURE 3. Effect of differential DC stimulation on T cell activation in vitro. Following culture in the presence of the indicated cytokine combinations, DC were compared in their capacity to stimulate allogeneic T cell proliferation. Day 5 DC were stimulated for 48 h with 200 U/ml GM-CSF and 20 ng/ml IL-4 only (■) or additionally with 5 ng/ml TNF- α (◆), 20 ng/ml TNF- α (●), 80 ng/ml TNF- α (▲), or with 6 μ g/ml ODN-1826 (■). At day 7, DC were mixed with T cells at the indicated ratios. One representative experiment of three is shown.

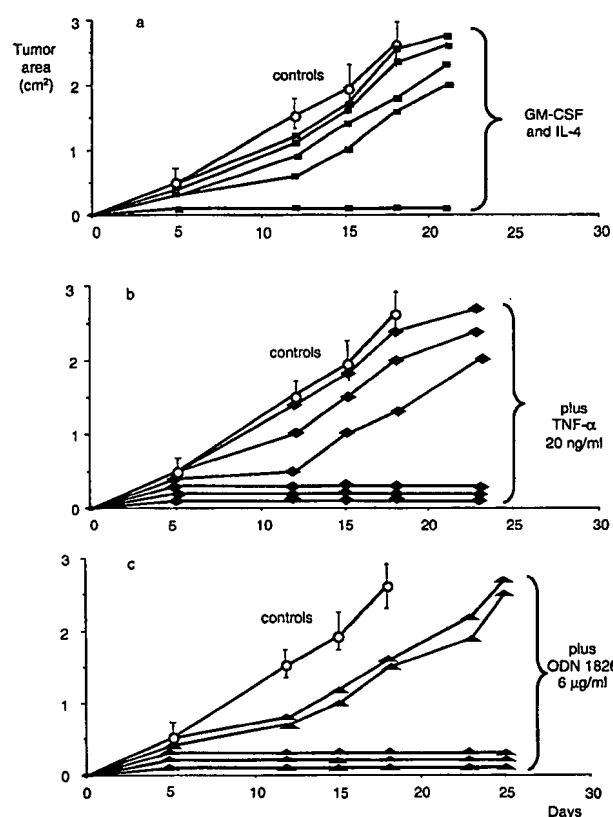


FIGURE 4. Prophylactic vaccination with differentially stimulated coculture cells. DC were cocultured with irradiated tumor cells for 4 days in the presence of 200 U/ml GM-CSF and 20 ng/ml IL-4 without further stimulation (*a*, ■) or additionally stimulated for 48 h with 20 ng/ml TNF- α (*b*, ◆) or 6 μ g/ml ODN-1826 (*c*, ▲). Coculture cells (1×10^6) were injected s.c. into the right flank. Seven days later, mice were challenged with 5×10^5 tumor cells. One representative experiment of three ($n = 45$) is shown ($p < 0.001$). Each curve represents one mouse except for the top curve (○), which is the mean \pm SEM of untreated control mice ($n = 5$).

tumor progression in the remaining 50% of mice and prolonging their survival to day 23 (Fig. 4*b*, $p < 0.001$). Immunization with ODN-1826-stimulated DC cocultured with tumor cells completely protected 59% of mice and delayed tumor growth in the remaining mice, increasing their survival up to day 25 (Fig. 4*c*, $p < 0.001$). In a preliminary study, we observed that the tumor vaccination potential can be further increased by two prophylactic immunizations before tumor challenge (data not shown).

DC vaccination induces tumor-specific protection

To evaluate the tumor specificity of DC vaccination we also performed control experiments with a different BALB/c-derived tumor line (Renca). Two independent experiments at $n = 30$ each were performed, and one representative with five mice per group is shown in Fig. 5. Tumor growth after injection of 5×10^5 Renca cells in unvaccinated mice (control group) was also rapid, leading to death by day 23 in all mice. Renca cells (5×10^5) were injected s.c. 7 days after vaccination with ODN-1826-stimulated DC cocultured with Colon-26 cells; all mice in the group ($n = 10$) finally succumbed to tumors (Fig. 5*a*, $p = 0.290$). However, 6 of 10 mice challenged with Renca cells after prophylactic vaccination with Renca cells cocultured with DC did not show any tumor formation (three of five mice for the experiment shown in Fig. 5*b*, $p < 0.001$). Injection of 5×10^5 Colon-26 cells 7 days after vaccination with DC cocultured with Renca cells led to lethal tumor

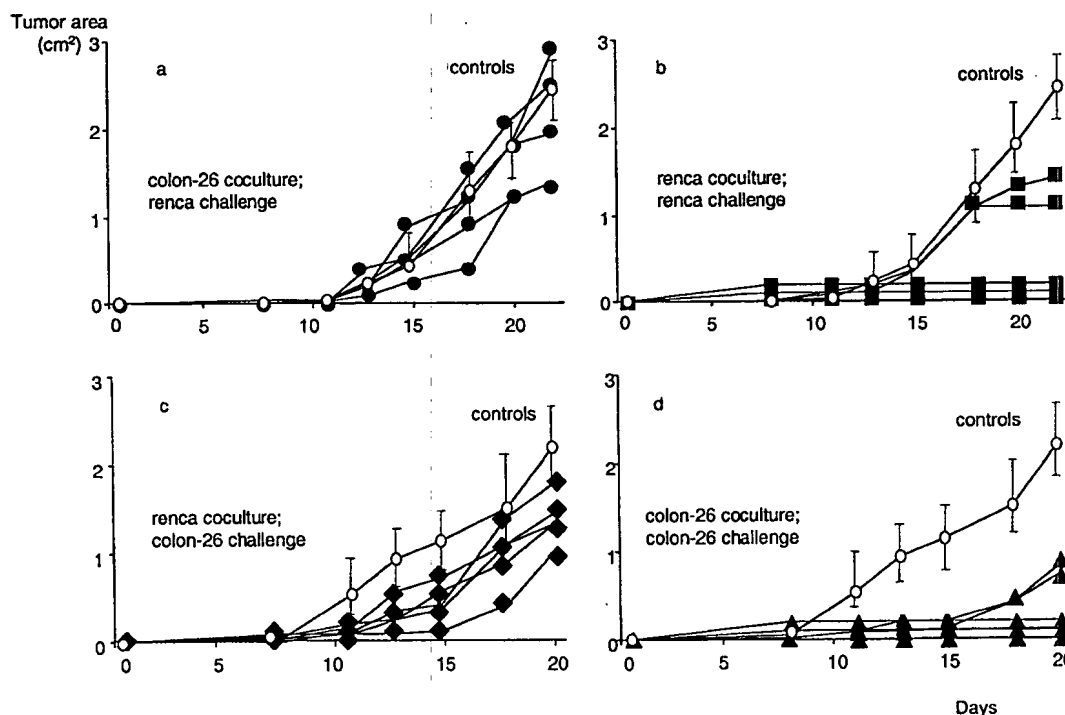


FIGURE 5. Tumor specificity of vaccination with DC after coculture with tumor cells. DC were cocultured with irradiated Colon-26 cells (*a* and *d*) or Renca cells (*b* and *c*) for 4 days in the presence of 200 U/ml GM-CSF and 20 ng/ml IL-4 and additionally stimulated for 48 h with 6 μ g/ml ODN-1826. Renca cells (5×10^5) were injected 7 days after vaccination with ODN-1826-stimulated DC cocultured with Colon-26 cells \bullet , (*a*, $p = 0.290$) or in mice vaccinated with ODN-1826-stimulated DC cocultured with Renca cells (*b*, \blacksquare , $p < 0.001$). Tumor growth after injection of 5×10^5 Renca cells in unvaccinated mice (control group \circ) was rapid, leading to death at day 23. In addition, 5×10^5 Colon-26 cells were injected 7 days after vaccination with ODN-1826-stimulated DC cocultured with Renca cells (*c*, \blacklozenge , $p = 0.250$) or ODN-1826-stimulated DC cocultured with Colon-26 cells (*d*, \blacktriangle , $p < 0.001$). One representative experiment of two ($n = 60$) is shown. Each curve represents one mouse except for the top curve (\circ), which is the mean \pm SEM of untreated control mice ($n = 5$).

development in all mice (Fig. 5*c*, $p = 0.250$). In contrast, 6 of 10 mice challenged with Colon-26 cells after prophylactic vaccination (Colon-26) remained tumor free (three of five mice for the experiment shown in Fig. 5*d*, $p < 0.001$). In addition, injection of 5×10^5 Renca cells 10 wk after complete rejection of Colon-26 tumors (following prophylactic vaccination with ODN-1826-stimulated DC cocultured with Colon-26 cells) led to s.c. formation of tumors in all mice ($n = 10$, data not shown). This indicates maintained specificity even after immunization-based rejection has occurred.

Therapeutic effect of ODN-1826-stimulated DC cocultured with tumor cells

As ODN-1826-stimulated DC cocultured with tumor cells were the most effective prophylactic tumor vaccine, we next evaluated their therapeutic potential in three independent experiments ($n = 40$). Tumors were induced by s.c. injection of 1 or 5×10^5 Colon-26 cells followed by ipsilateral injection of 10^6 cocultured cells 3, 7, or 10 days later. One representative result with five mice per group is shown in Fig. 6. In the untreated control mice, no tumors were visible 3 days after tumor challenge but became visible (0.3–0.5 cm diameter) at day 7 (see also Figs. 4 and 8*a*). These mice die or have to be euthanized around day 18. As a mean of all experiments, vaccination 3 days after injection of 1×10^5 tumor cells completely prevented tumor growth in 40% (two of five mice in Fig. 6*a*, $p < 0.05$). In the remaining three mice, tumor size at day 18 was reduced, concomitant with a prolonged survival up to day 29 ($n = 2$) and beyond 32 days ($n = 1$). Vaccination 3 days after s.c. injection of 5×10^5 tumor cells (Fig. 6*b*, $p < 0.05$) prevented tumor growth in one of five mice, and reduced tumor growth in the

remaining mice prolonging survival up to day 23. Even when mice were vaccinated 10 days after injection of 1×10^5 tumor cells, reduced tumor growth and prolonged survival was observed (Fig. 6*c*, $p < 0.05$). Thus, the time window for inducing tumor immunity was 10 days after tumor cell injection. For the high tumor challenge (5×10^5 cells), no prevention of tumor growth and only a trend toward prolonged survival up to day 20 (in three of five mice) following tumor vaccination 7 days after tumor challenge was observed (Fig. 6*d*, $p = 0.371$).

DC vaccination prolonged tumor immunity

To study the persistence of tumor immunity, mice that had not developed tumors after prophylactic or therapeutic vaccination were rechallenged 6 wk after the first vaccination by s.c. injection of 5×10^5 tumor cells. As shown in the representative experiment of Fig. 7 independent of the initial vaccination protocol used, no animal (of a total of 20 animals rechallenged) developed a tumor. This suggests that vaccination with DC cocultured with tumor cells mediates tumor protection lasting for at least 6 wk. In addition, no tumors formed even after repeated tumor challenges (data not shown).

Adjuvant effect of ODN-1826 on growth of Colon-26-induced tumors

Our findings demonstrated that ODN-1826 enhanced the T cell-activating capacity of DC in vitro. This prompted us to examine whether in vivo the prophylactic vaccination using ODN-1826 (without DC) could influence subsequent tumor development.

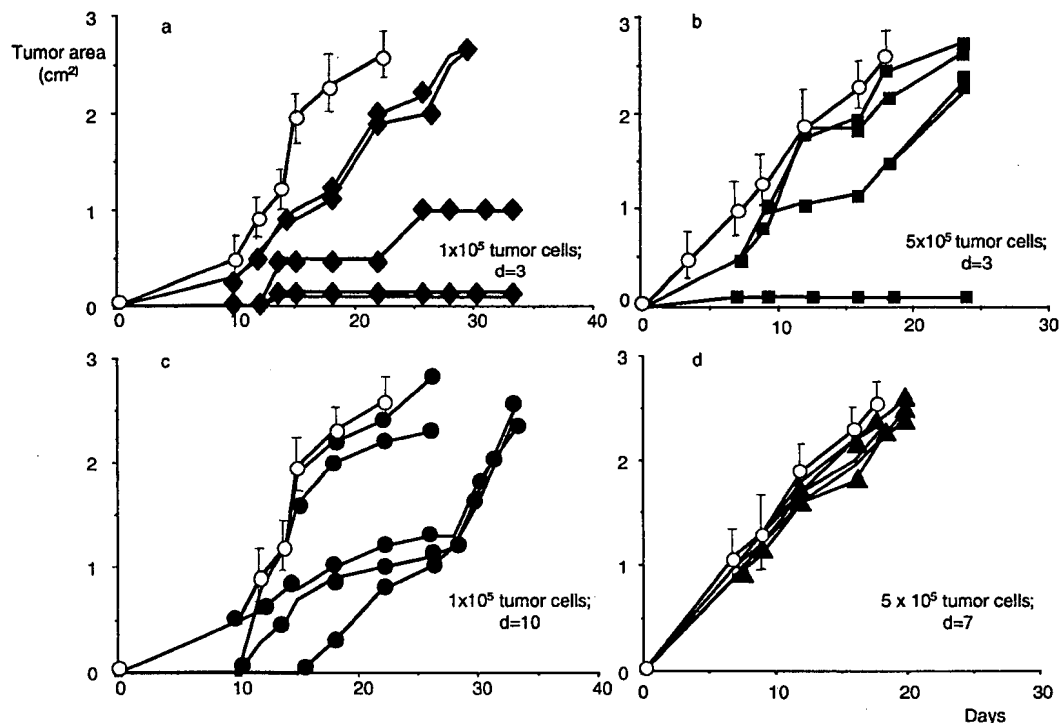


FIGURE 6. Therapeutic vaccination with ODN-1826-stimulated coculture cells. Coculture cells (1×10^6) stimulated with ODN-1826 were injected s.c. into the right flank 3 days after tumor challenge with 1×10^5 tumor cells (a, \diamond , $p < 0.05$) or 5×10^5 tumor cells (b, \blacksquare , $p < 0.05$), 10 days after tumor challenge with 1×10^5 tumor cells (c, \bullet , $p < 0.05$) or 7 days after injection of 5×10^5 tumor cells (d, \blacktriangle , $p = 0.37$). One representative experiment of three ($n = 45$) is shown. Each line represents one mouse except for the top line (\circ), which is the mean \pm SEM of untreated control mice ($n = 5$).

ODN-1826 was injected s.c. with or without irradiated tumor cells and, after 7 days, mice were challenged s.c. with 5×10^5 vital Colon-26 cells. Fig. 8 shows one representative experiment of three ($n = 30$). Control mice developed rapidly growing tumors with no mice surviving day 18 (Fig. 8a). Prophylactic injection of ODN-1826 without irradiated tumor cells resulted in a delayed tumor growth but no survival was observed beyond day 20 (Fig. 8b, $p = 0.173$). ODN-1826 coinjected with irradiated tumor cells protected a small proportion of mice (25% for all experiments) and reduced tumor progression in most of the remaining animals, prolonging survival up to day 25 (Fig. 8c, $p < 0.05$). Injection of irradiated tumor cells alone resulted in no protection (data not shown).

Discussion

Using three function-related *in vitro* assays and a murine tumor model, we compared DC stimulated in the presence of GM-CSF and IL-4 alone to DC additionally stimulated with TNF- α or with the CpG motif-containing oligonucleotide ODN-1826 (34). We demonstrate that the extent of DC maturation, as reflected by surface density of costimulatory and MHC II molecules, correlated with the secretion of IL-12, with the T cell-activating potential *in vitro*, and with the induction of tumor immunity *in vivo*. ODN-1826 was identified as a very potent DC activator, clearly superior to the well-established DC stimulator TNF- α (5, 27, 35).

For enrichment of DC from bone marrow, we modified the protocol published by Inaba (33) by additionally supplementing the culture medium with IL-4. In line with published data, this gave consistently better yields in DC and suppressed the development of monocytes (36). As IL-4 is the key inducer of Th2 responses and antagonizes proinflammatory IL-12, we ruled out that IL-4 inhibits IL-12 production by DC. DC secreted stable baseline amounts of IL-12 independent of the IL-4 concentration used (Fig. 2). This is

concordant with the notion that the antagonism of IL-12 and IL-4 is not due to a direct suppressive effect on each other's synthesis (37–39).

Analyzing the effect of differential DC stimulation on IL-12 production, we found that ODN-1826 was the most potent stimulator. Its effect exceeded that of TNF- α by a factor of 5, which by itself was 4-fold more potent than baseline stimulation. DC cultured in the presence of GM-CSF and IL-4 alone expressed moderate levels of the costimulatory molecules CD80 and CD86, of the DC activation marker CD40, of the adhesion integrin CD54 (ICAM-1), and of the Ag-presenting proteins MHC class I and MHC II. TNF- α at its optimal concentration up-regulated DC markers MHC II, CD86, and CD80 1.3- to 3-fold. Compared with baseline stimulation, ODN-1826 induced an increase in mean expression of CD80, CD86, and MHC II by a factor of 4, 2, and 6, respectively. Examining the effect of DC maturation on *in vitro* T cell proliferation, we found a maturation-dependent T cell-activating potential of the respective DC preparations. Especially at low numbers, ODN-1826-activated DC induced the highest T cell proliferation, whereas TNF- α -stimulated DC again showed an intermediate effect. This finding may be of particular relevance *in vivo*, where DC are scarce in peripheral blood, tissue, and lymph nodes. Our data further imply that DC have to be strongly stimulated to raise a productive T cell response. We propose that this stimulation can be caused either by strong signals like bacterial DNA or by combinations of weaker signals such as TNF- α plus CD40 ligation.

DC activating potential for ODN-1826 was described by Vogel and coworkers who showed that this oligonucleotide was nearly as effective as LPS in activating C57BL/6 fetal skin-derived DC to express MHC II, costimulatory, and accessory molecules and to stimulate T cell proliferation (9). In addition, it was about 10 times more potent than LPS in stimulating IL-12 secretion. Vogel et al. did not compare it to other stimuli such as TNF- α or IL-4. Using

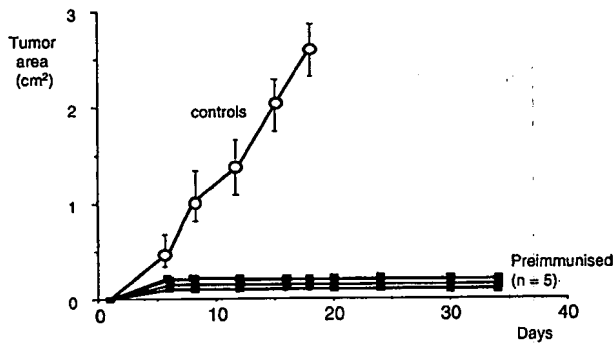


FIGURE 7. Tumor vaccination induces long lasting tumor immunity. Untreated mice (○) or mice that had demonstrated complete tumor protection after prophylactic vaccination (■) were rechallenged with 5×10^5 tumor cells 6 wk after vaccination. One representative experiment of six is shown ($p < 0.001$).

the control ODN-1982, we could not observe any stimulatory effects on the surface expression of MHC II, CD86, and CD80, the IL-12 secretion or the T cell activation of DC in vitro. This is completely in line with previous reports of control ODN, in which the CpGs were methylated or, inverted, lacked these stimulatory activities, confirming that the stimulatory effects of ODN-1826 were dependent on its unmethylated CpG motifs (Refs. 9, 15; and A. M. Krieg, unpublished observations).

Of particular interest was the observation that the expression of DC activation and maturation markers correlated with the IL-12 synthesis and the capacity to stimulate T cell proliferation. For all parameters tested, ODN-1826 was the most potent stimulus, TNF- α had an intermediate effect, and GM-CSF and IL-4 alone showed the weakest effects. Therefore, a common intracellular pathway underlying DC activation and regulating the cellular aspects required for T cell activation seems to be triggered by different maturation-inducing stimuli. Recently, it has been shown that Toll-like receptors are evolutionarily conserved immune response-activating molecules and are expressed by DC, monocytes, and various other mammalian cell types (40). Toll-like receptor 2 was identified as the signal-transducing receptor for LPS, triggering a signal cascade that leads to activation of NF- κ B (41) and c-Jun NH₂-kinase (42, 43). NF- κ B activation is central to the process of DC maturation (44) and NF- κ B is activated by CpG-ODN in lymphoma cells (34) and macrophages (45, 46). It is not known whether Toll-like receptors also participate in the activation of DC by inflammatory signals other than LPS but it seems likely that the efficacy by which NF- κ B is activated by the respective signal cascade determines the degree of DC maturation.

A key finding of this work is that matured DC after coculture with tumor cells were able to confer tumor-specific protection even in the BALB/c mouse strain, which is prone to raise a Th2 response (32). Using the aggressively growing colon carcinoma line Colon-26, which leads to euthanasia around day 18, allowed us to investigate the limits of DC-based tumor vaccination and to assess differences in the potency of the respective DC preparations. Vaccination with ODN-1826-stimulated DC cocultured with tumor cells prevented the development of tumors in ~60% of mice. Preliminary findings of ongoing studies in our laboratory indicate that two vaccinations with ODN-1826-stimulated DC are even more protective, conferring tumor resistance to about 80% of mice. ODN-1826-stimulated DC were also therapeutically effective in this model, preventing tumor formation in up to 40% of mice (Fig. 5). Thus, even in a Th2-biased mouse strain, a high level of protection against tumor challenge can be induced. This extends a

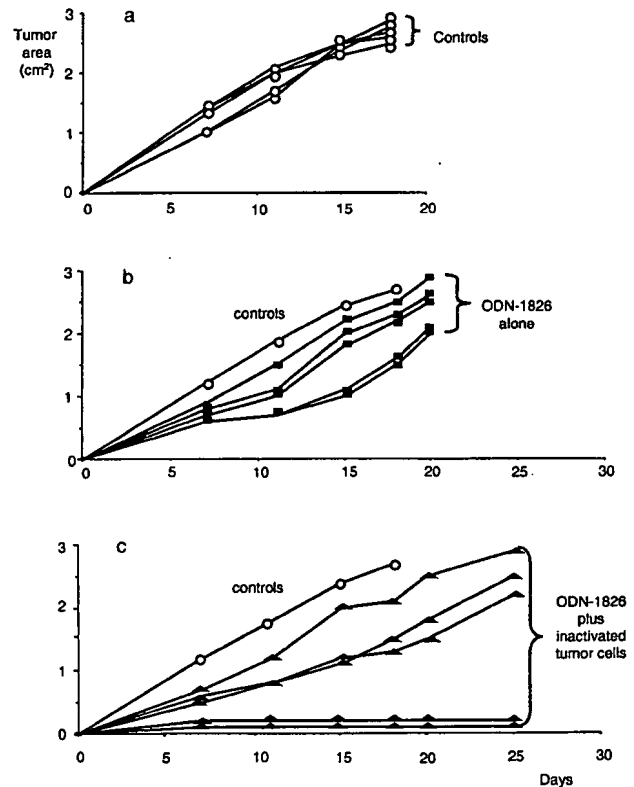


FIGURE 8. Vaccination with ODN-1826 without DC. Untreated mice (a, ○) died at day 18. Single s.c. injections of 50 μ g/ml ODN-1826 alone (b, ■, $p = 0.173$) or as adjuvant for inactivated tumor cells (c, ▲, $p < 0.05$) were given to five mice per group. Seven days later, mice were challenged with 5×10^5 tumor cells ipsilaterally. Each curve represents tumor formation of one mouse. The mean tumor size of control mice (a) is shown as reference (○) in b and c. One representative experiment of three ($n = 30$) is shown.

recent study comparing different CpG-containing oligonucleotides in their ability to act as adjuvants in the induction of a Th1 response. ODN-1826 was identified as the most potent one (17).

In correlation to their maturation status, DC stimulated with TNF- α or GM-CSF plus IL-4 alone and cocultured with tumor cells were less effective as prophylactic tumor vaccines and, also, ODN-1826-stimulated DC did not induce complete tumor immunity. At first glance, this seems to be at variance with studies published by the groups of Kufe and Mayordomo, who reported complete protection even though DC were stimulated only with GM-CSF (20) or with GM-CSF and IL-4 (21). We propose that this discrepancy may be due to the rather slow tumor growth induced by the cell lines used in both studies (MC-38, B16, and 3LL led to death between 28 and >60 days) and/or due to the Th1 bias of C57BL/6 mice (28). Irrespective of its reason, this discrepancy highlights the importance of thoroughly investigating different models before generalized conclusions on DC-based tumor vaccination for the application in man can be drawn.

First clinical studies on DC-based tumor vaccination have been performed in patients with B cell lymphoma (29), melanoma (31), and prostate carcinoma (30). These studies achieved some clinical benefit (partial or complete responses), which was, however, limited to a rather small proportion of patients (~25%). The observations of this study suggest that one reason for the limited success of the vaccination trials may reside in insufficient means to induce DC maturation. Because even the strongest stimulation of DC did not confer complete protection against aggressively growing tumors, other strategies are required to eventually maximize the clinical benefit of DC-based tumor vaccination.

In a last set of *in vivo* experiments, we evaluated the injection of ODN-1826 as an adjuvant. When coadministered with inactivated tumor cells, it retarded the growth of subsequently injected colon carcinoma cells and in 25% of mice it led to complete protection from tumor growth. Other bacterial products are already approved or in clinical trials as adjuvants for tumor vaccines. The mycobacterial preparation bacillus Calmette-Guérin (which contains bacterial DNA with CpG motifs) has been approved for immunotherapy of urothelial cancer (47). Furthermore, it has been studied in patients who were tumor free following surgery for colon carcinoma. Bacillus Calmette-Guérin combined with autologous tumor cells reduced the recurrence rate by 44% in patients with stage II colon carcinoma resulting in a trend toward prolonged survival (48). For patients with stage III carcinoma, no clinical benefit was noted. Other adjuvants are currently in clinical trials, i.e., the saponin formulation QS21 and the LPS derivative monophosphoryl lipid A (MPL) (49, 50). Because IL-12 improves the efficacy of QS21 and MPL (51), and CpG-ODN induces high amounts of IL-12, we propose that a combination of QS21, MPL, and CpG-ODN may improve antitumor responses even further.

In summary, we have demonstrated that surface expression of DC markers, IL-12 secretion, and T cell proliferation are coordinately enhanced during DC maturation. ODN-1826 stimulated this process most potently, whereas TNF- α showed an intermediate effect compared with basal stimulation with GM-CSF and IL-4 alone. In correlation with the *in vitro* stimulation, we found that, likewise, the induction of tumor immunity depends upon the extent of DC maturation. We conclude that *in vitro* maturation of DC is an efficient strategy to improve tumor vaccination protocols.

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Oligonucleotide Adjuvants for T Helper 1 (Th1)-specific Vaccination

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T helper cell 1 (Th1)-dependent delayed hypersensitivity reactions are an important part of host defenses against intracellular infections. Yet, more than two centuries after Jenner's successful inoculation against smallpox, we still do not know exactly how to produce safe vaccines that stimulate Th1 immunity. Specialized bone marrow-derived antigen-presenting cells normally are required to initiate all T cell-dependent immune responses (1). However, in order for Th responses to shift to a Th1 phenotype, interleukin (IL)-12 needs to be present at the time of antigen recognition (2). IL-12 drives natural killer (NK) and Th1 cells to generate interferon (IFN)- γ , that subsequently impels macrophages to initiate delayed hypersensitivity reactions. IFN- γ also inhibits the synthesis of IL-4 and IL-5 by Th2 cells (3). In the absence of IL-12 induced IFN- γ production, Th2 responses usually dominate.

But how do particular infectious agents induce IL-12 release? Accumulating evidence indicates that immunostimulatory CpG sequences (ISS) in the DNA of bacteria may be one of the major IL-12-inducing factors (4). Complete Freund's adjuvant, composed of killed mycobacteria dispersed in mineral oil, is an established inducer of Th1-dependent delayed hypersensitivity reactions. More than 10 years ago, Tokunaga and coworkers discovered that DNA purified from mycobacteria fostered the release of IFN- γ by mouse NK cells (5). Fractionation of the DNA led to the isolation of several different short palindromic sequences, most of them centered around a CpG dinucleotide core, that had direct NK stimulatory activity (6). Subsequent studies showed that synthetic phosphodiester or phosphothioate oligodeoxynucleotides, which reproduced the immunostimulatory DNA sequences from mycobacteria, could activate NK cells and induce B lymphocyte proliferation *in vitro* (6-9). Methylation of cytosine residues in the bacterial DNA or in the corresponding oligodeoxynucleotides destroyed their immunostimulatory activities (7).

During early investigations of DNA vaccination, we observed that nonspecific bacterial DNA enhanced immune responses to a coinjected antigen expression vector (10). Naked DNA immunization stimulated a selective Th1 immune response that persisted upon secondary challenge with protein antigen (11). In some instances, the Th1 skewing effects of gene vaccines could be manipulated by changing the number of immunostimulatory sequences in the plasmid DNA backbone (12). Simple coinjection of bacterial DNA or immunostimulatory oligodeoxynucle-

otides with a DNA vaccine or with representative protein antigens also promoted antigen-specific Th1 responses (13, 14), even in mice with preexistent Th2 immunity. Incubation of purified human macrophages with bacterial DNA, or with immunostimulatory CpG oligodeoxynucleotides, stimulated the production of IL-12, IL-18, and IFN- α (14).

Now, Chu et al. have shown that vaccination of mice with an antigen and an immunostimulatory CpG oligodeoxynucleotide in incomplete Freund's adjuvant induced a powerful Th1 immune response, comparable to that achieved by coinjection of the antigen in complete Freund's adjuvant (15). In contrast, mice vaccinated with antigen and control oligodeoxynucleotides lacking the CpG motif, developed a skewed Th2 type immune response.

An efficacious vaccine must be devoid of systemic toxicity. Systemically administered immunostimulatory oligodeoxynucleotides can trigger a cytokine syndrome in mice, characterized by TNF release, hypotension, and shock (16). Although exogenous IL-12 induces potent Th1 immune responses, high concentrations of the cytokine also can be harmful to the recipient (17). The potential side effects of immunostimulatory CpG sequences could be reduced by including them in the backbones of DNA vaccines, or by tethering the immunostimulatory oligodeoxynucleotides directly to precipitated antigens.

We still do not understand how mouse macrophages, B lymphocytes, and NK cells recognize specific DNA sequences in bacterial DNA. The immunostimulatory CpG motifs could theoretically bind to complementary sequences in DNA or mRNA. More likely, the unmethylated CpG core interacts with one or more signal transduction molecules in the cytoplasm, or on the plasma membrane. An analogous system mediates the induction of IFN- α synthesis by double stranded viral RNA (18).

In the future, it may be possible to skew the immune response to vaccination to a Th1, Th2, or a mixed Th1/Th2 outcome, simply by titering the concentrations of coadministered immunostimulatory CpG oligodeoxynucleotides. Th1 vaccines should be particularly useful for the prevention and treatment of allergic diseases and asthma, since the IFN- γ released by Th1 lymphocytes and NK cells can down-regulate IgE synthesis, as well as inhibit Th2 cells that control the late phase component of the allergic response. It is conceivable that the coadministration of an immunostimulatory oligodeoxynucleotide sequence with a weak tumor antigen could stimulate a delayed hypersensitivity response

sufficient to eliminate malignant cells. By increasing endogenous IFN- γ synthesis, therapeutic Th1 vaccines could promote recovery from chronic viral or parasitic infections.

In summary, immunostimulatory oligonucleotides are adjuvants that simplify Th1 induction in experimental sys-

tems. Their applications in clinical immunology will depend on whether the data generated in murine models will be reproducible in humans and whether the side effects of cytokine overproduction will be acceptable.

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Bacterial DNA-Induced NK Cell IFN- γ Production Is Dependent on Macrophage Secretion of IL-12

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Bacterial DNA (bDNA) activates B cells and macrophages and can augment inflammatory responses by inducing release of proinflammatory cytokines. We found that bDNA stimulation of mouse spleen cells induced NK cell IFN- γ production that was dependent upon the presence of unmethylated CpG motifs, and oligonucleotides with internal CpG motifs could also induce splenocytes to secrete IFN- γ . The bDNA-induced IFN- γ response was strictly macrophage dependent. While splenocytes from SCID mice secreted IFN- γ in response to bDNA, depletion of macrophages eliminated this response. Additionally, purified NK cells did not respond to bDNA; however, addition of macrophages restored the NK cell IFN- γ response. Coculture of NK cells with preactivated macrophages further increased bDNA-induced NK cell IFN- γ production. Anti-IL-12 or IL-10 inhibited bDNA-induced IFN- γ response. Treatment of purified macrophages with bDNA resulted in IL-12 secretion accompanied by an increase in IL-12 p40 mRNA level. Although isolated NK cells did not make IFN- γ in response to bDNA, NK cells costimulated with IL-12 gained the ability to respond to bDNA. These experiments show that bDNA induces macrophage IL-12 production which, in turn, stimulates NK cell IFN- γ production. Macrophage-derived IL-12 renders NK cells responsive to bDNA permitting an even greater IFN- γ response to bDNA. © 1997 Academic Press

INTRODUCTION

Interferon- γ (IFN- γ) is a T cell/NK-cell-derived cytokine that plays a critical role in the regulation of inflammatory responses, including host protection from a number of infectious agents (1–8). In contrast to its beneficial actions, IFN- γ also can serve as an initiating factor in the frequently lethal sepsis syndrome (9, 10). Although T cell production of IFN- γ can be induced after recognition of specific antigens by T cells, NK cell production of IFN- γ is not antigen-specific and can be driven by macrophage/dendritic cell-derived IL-12 (11–

13). IL-12 production is induced by a number of proinflammatory agents (including LPS and IFN- γ) and can be strongly inhibited by IL-10 (14, 15). Thus, the relative contributions of IL-12 and IL-10 may determine whether an IFN- γ -based inflammatory response is sufficient to combat an infection or is excessive to the extent that the host is harmed.

Microorganisms elicit inflammatory responses by the direct interaction with cells of the immune system (such as macrophages) or by the release of soluble mediators (such as endotoxin or enterotoxin) which induce host cytokine production. Bacterial DNA is known to activate B cells (16, 17) and has been shown to induce IFN- γ production *in vitro* and *in vivo* (18, 19). Additionally, the efficacy of DNA-based immunization is markedly enhanced by the presence of immunostimulatory CpG motifs within the immunizing vector (20). Our laboratory has examined the proinflammatory properties of bacterial DNA (bDNA), and we previously showed that bDNA functioned as a potent stimulator of *in vivo* IFN- γ production by NK cells (but not T cells). *In vivo* exposure to as little as 30 μ g of bDNA also markedly augmented the toxicity of endotoxin (21). To begin to unravel the mechanism by which bDNA induces cytokine production, we have examined cellular interactions responsible for the host response to bDNA. We examined the *in vitro* response of defined lymphoid populations to bDNA, and, unexpectedly, we found that isolated NK cells do not secrete IFN- γ following challenge with bDNA. However, NK cells do produce IFN- γ in response to bDNA if macrophages are present in the culture. Additionally, the requirement for macrophages can be replaced by exogenous IL-12. These studies indicate that macrophages are one of the principal cells activated by bDNA. Activation of macrophages by bDNA induces a substantial IL-12 response which is responsible for driving the NK cell IFN- γ response (and potentially inducing other proinflammatory cytokines). These findings are relevant to the management of deleterious inflammatory responses and to the development of DNA-based vaccines and adjuvants.

MATERIALS AND METHODS

Mice. All experiments were performed using spleen cells derived from 8- to 20-week-old, virus antibody-free, female C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, ME). This strain expresses the NK1.1 antigen on the surface of NK cells (22). C57BL/6 SCID mice (Jackson) were generously provided by Dr. Zuhair Ballas (University of Iowa).

DNA preparations. *Escherichia coli* (EC) and calf thymus (CT) DNA were purchased from Sigma Chemical Company (St. Louis, MO). DNA was dissolved in 0.15 M NaCl at a concentration of 1 mg/ml and heated to 100°C for 1 hr and rapidly cooled. Both DNA preparations were tested for endotoxin contamination using the Limulus amoebocyte lysate assay (23). EC DNA contained 37 ng of endotoxin/mg of DNA and CT DNA contained 6 ng of endotoxin/mg of DNA. We were able to accurately quantitate LPS that we deliberately added to DNA solutions, indicating that the DNA did not interfere with the Limulus assay. DNA was selectively methylated at CpG motifs using 2 U CpG methylase (New England Biolabs)/ μ g DNA. Methylated DNA was reextracted with chloroform methanol and found to be completely resistant to *Hepa II* (specific for unmethylated CCGG) but could be cut by *MspI* which can cleave methyl-CpG.

Oligodeoxynucleotides (ODN). Nuclease-resistant phosphorothioate ODN were purchased from Midland Certified Reagent Company (Midland, TX). The LPS level in ODN was less than 12.5 ng/mg. The sequences of phosphorothioate (ODN) used were: 1826, 5'TCC-ATGACGTTCTGAGCTT3'; 1668, 5'TCCATGACG-TTCCTGATGCT3'; 1745, 5'TCCATGAGCTTCCTG-AGTCT3'; and 1642, (5'TGC3')₇. ODN 1826, 1668, and 1745 differ mainly with respect to the presence of an internal CpG (1826 and 1668) that is switched to GpC in 1745. ODN 1826 also has a CpG motif proximal to the 3' end.

Cytokines. Recombinant murine IL-10 and IL-12 were purchased from R & D Systems (Minneapolis, MN). Recombinant murine IFN- γ was generously provided by Genentech.

Flow cytometry. The following fluorochrome-tagged monoclonal antibodies were used in positive and negative selection experiments: anti-NK1.1 (BTK 136, Ref. 24); anti-CD4 (mAb 2B6, Ref. 25); anti-CD8 (mAb 53.6.71, Ref. 26); anti-B220 (mAb 6B2, Ref. 27); anti-Mac 1 (mAb M1/20.15, Ref. 28). All cell sorting used a Coulter Epics 753 flow cytometer. Lymphocytes were sorted based on cell surface phenotype, and T cell + B cell subpopulations were >98% pure. Macrophages were collected initially as plastic adherent cells. Plates

were gently rinsed with warm complete RPMI, and adherent cells were harvested by vigorous flushing with ice-cold PBS. The enriched cells were then stained with mAb N418 (anti-CD11c, which identifies splenic dendritic cells) (29), anti-B220, and anti-Mac-1 and purified with a Coulter EPICS 753 cell sorter. Macrophages were identified as B220⁻ Mac1^{bright} N418^{dull} (29). Cells designated as FACS-purified macrophages were >95% Mac1^{bright}, and cells designated as purified NK cells contained <2% contaminating T or B cells.

Cell cultures. In all experiments, lymphoid cells were cultured on Elispot wells previously coated with anti-IFN- γ (see below). Unfractionated or negatively selected spleen cells were cultured at 5×10^5 – 10^6 /ml in a volume of 0.2 ml. Purified NK cells were cultured at a concentration of 2000 cells/0.2 ml and costimulating cells were added back to the cultures (300–10,000 cells/well). Cultures were stimulated for 24 hr with bDNA, calf thymus DNA (CT DNA), or synthetic oligonucleotides. Culture plates were washed and Elispots were subsequently developed. In some experiments, adherent cells were preactivated with IFN- γ (100 u/ml) for 1 hr and washed five times prior to FACS isolation of Mac 1⁺ cells. These cells, as well as unactivated macrophages, were later cocultured with purified NK cells. To determine the role of IL-12 in the NK cell response to bDNA, we added a cocktail containing 10 μ g each of anti-IL-12 mAbs C.15.1.2, C17.15, C17.8, and C15.6 originally isolated by Dr. G. Trinchieri (Wistar Institute).

IFN- γ and IL-12 elispot assay. Spleen cells from treated mice were plated onto Immulon 2 plates that were first coated with 3 μ g/ml of monoclonal antibody to IFN- γ (R4-6A2, Pharmingen) or anti-IL-12 (mAb 17.8) in 0.05 M carbonate buffer, pH 8.2, and incubated for 18 hr and then blocked with PBS/10% FCS. Elispots were developed as previously described (30) by the sequential addition of polyclonal rabbit anti-mouse IFN- γ (produced in our laboratory) or goat anti-IL-12 (R&D Systems, Minneapolis, MN). Following overnight incubation, IL-12 plates were treated with biotin-rabbit anti-goat IgG. The development of either Elispot assay was accomplished by the sequential addition of biotin-conjugated donkey anti-rabbit IgG, alkaline phosphatase-streptavidin, substrate (5-bromo-4 chloro-3-indoyl phosphate, Sigma) dissolved in 0.6% agarose in AMP substrate buffer. Elispot-forming cells are directly counted under 10 \times magnification using a dissecting microscope.

IL-12 RT-PCR. Cells were cultured 2 hr with complete RPMI alone, EC DNA (3 μ g/ml), or CT DNA (3 μ g/ml). Cells were harvested and lysed in RNazol B (Tel-Test, Inc., Friendswood, TX) according to manu-

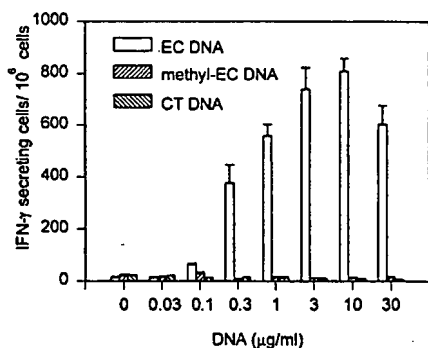


FIG. 1. Identification of IFN- γ -secreting cells following *in vitro* stimulation with DNA stimulation. Spleen cells (10^5 /well) were cultured for 24 hr on anti-IFN- γ -coated plates in the presence of EC DNA, methyl-EC DNA, or CT DNA at the concentrations indicated. The number of IFN- γ -producing cells was determined by the Elispot assay. The averages \pm SE of triplicate cultures are shown.

facturer's directions for the purification of total RNA. Total RNA was warmed to 70°C for 5 min and then reverse transcribed into cDNA in 20 μ l in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM NTPs, 200 u M-MLV-RT, 40 u RNasin, and 200 ng oligo(dT) for 1 hr at 37°C. The cDNA was amplified by PCR using 8 μ l of the cDNA preparation in a 50- μ l reaction mixture containing 2 U of *Taq* polymerase in 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM NTPs, and 25 pM of each primer. The following primers were used for PCR amplification: IL-12 p40 sense (position 183-206), 5'-GTG ACA CGC CTG AAG AAG ATG ACA-3'; and antisense (position 637-617), 5'-CTC GGC AGT TGG GCA GGT GAC-3'; this amplifies a 455-bp PCR product. The HPGRT primers were: sense (position 514-538), 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3'; and antisense (position 652-678), 5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3'. This amplifies a 165-bp product.

RESULTS

Bacterial DNA induces *in vitro* production of IFN- γ . Our previous study showed that treatment of mice with bDNA induced a significant NK-cell-derived IFN- γ response (21). To define further the mechanism by which bDNA induces IFN- γ production, we examined the *in vitro* response of mouse spleen cells to bDNA. When unfractionated splenocytes were cultured with bDNA, we observed a strong IFN- γ response (Fig. 1). There was no significant IFN- γ response when cells were treated with CT DNA. We performed additional experiments to exclude the possibility that a contaminant in the bDNA was responsible for inducing IFN- γ production. The bDNA used in these experiments contained less than 40 ng of endotoxin/mg DNA. Thus, spleen cell

cultures challenged with 10 μ g bDNA were exposed to less than 0.5 ng of endotoxin, a concentration which did not induce IFN- γ production over background (data not shown). To exclude the possibility that trace amounts of other contaminants in the bDNA were responsible for IFN- γ induction, we examined the stimulatory capacity of DNA that had been previously treated with CpG methylase, and we found that methylation of cytosine (when present in CpG dinucleotides) also abolished the ability of bDNA to induce IFN- γ production (Fig. 1). To define further the structural properties of bDNA that are required for the induction of IFN- γ secretion, we examined two phosphorothioate-modified (nuclease resistant) oligonucleotides (1826 and 1668) containing internal CpG (and 3' CpG in the case of 1826) and compared the IFN- γ response to that elicited by phosphorothioate ODN 1745 in which the internal CpG is switched to GpC. Additionally, we evaluated the ability of a 21-mer repeating TGC trinucleotide (1642) to induce IFN- γ production. The results presented in Fig. 2 show that only oligonucleotides 1826 and 1668 were potent inducers of IFN- γ production. These results suggest that structural properties of bDNA perhaps related to internal CpG motifs are required for induction of IFN- γ . Additionally, these findings further argue against the possibility that a contaminant was responsible for stimulating IFN- γ production.

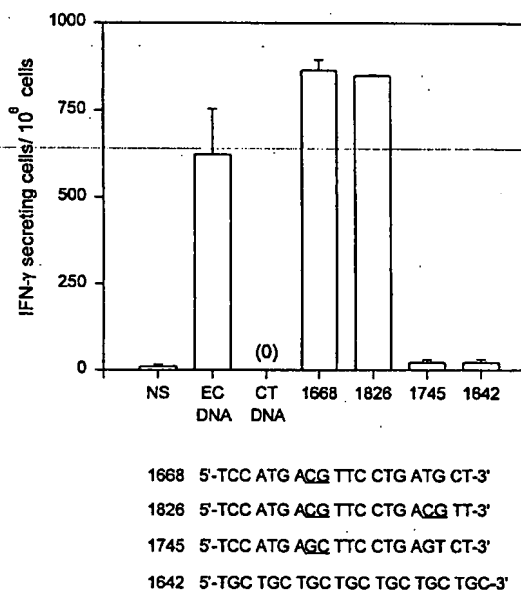


FIG. 2. IFN- γ is secreted in response to bDNA or oligonucleotides containing CpG. Spleen cells (10^5 /well) were cultured for 24 hr on anti-IFN- γ -coated plates in the presence of 1 μ g/ml DNA or ODN as indicated. The sequences of the ODN are described under Materials and Methods. The number of IFN- γ -secreting cells was determined by the Elispot assay. This is one of five similar experiments. NS, no stimulation.

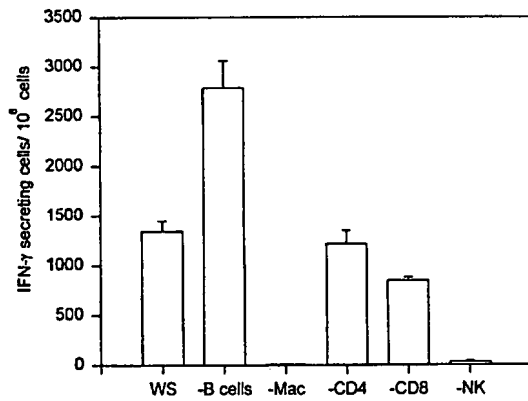


FIG. 3. bDNA induces macrophage-dependent IFN- γ secretion by NK cells. Spleen cells (WAS) were stained for B220, Mac 1, CD4, CD8, or NK1.1 and then depleted of these populations using flow cytometry. Negatively selected populations (10^5 /well) were cultured for 24 hr on anti-IFN- γ -coated plates with $10 \mu\text{g/ml}$ EC DNA. The number of IFN- γ -producing cells was determined by the Elispot assay. This is one of five similar experiments.

Bacterial DNA induces macrophage-dependent, NK cell IFN- γ production. To identify the bDNA responsive cell, we used a strategy of negative selection by cell sorting. The results presented in Fig. 3 show that depletion of CD4⁺, CD8⁺, or B220⁺ cells do not diminish the IFN- γ response of spleen cells; however, depletion of NK cells reduced the IFN- γ response by 95%. Cultures depleted of T cells or B cells often showed an increase in IFN- γ production (especially in the case of B cell depletion). This observation may be due to an absolute increase in the number of NK cells in cultures depleted of diluting T cells or B cells. Interestingly, depletion of macrophages also eliminated the IFN- γ response to bDNA. This latter finding suggested that bDNA stimulated NK cells by a macrophage-dependent mechanism. Additionally, bDNA stimulation of spleen cells from SCID mice resulted in a vigorous IFN- γ response that was abolished by depletion of Ia⁺ cells (Fig. 4). Collectively, these findings argue against T cell participation in the IFN- γ response to bDNA.

We confirmed the requirement for macrophages in the NK cell response to bDNA by culturing purified NK cells with FACS purified Mac 1⁺ adherent cells in the presence of bDNA. Figure 5 shows that adding back macrophages reconstituted the IFN- γ response to bDNA, and the effect of macrophages was strictly dependent on the number of macrophages in the culture. Coculture of NK cells with purified B cells or T cells did not result in IFN- γ production (data not shown). If macrophages were preactivated with recombinant IFN- γ (100 u/ml) for 1 hr prior to coculture with NK cells, the resulting IFN- γ response was approximately twice that seen in cultures containing unstimulated macrophages plus NK cells and approximated the IFN- γ re-

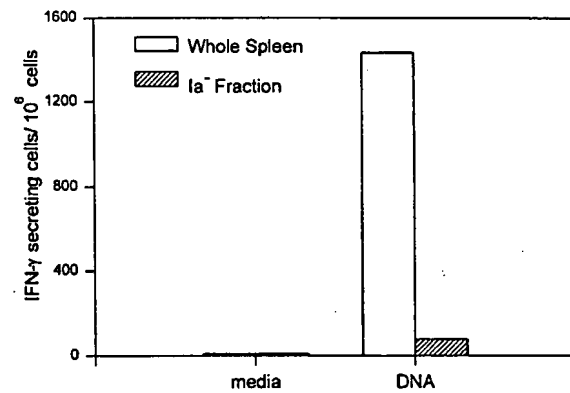


FIG. 4. bDNA-induced IFN- γ secretion by spleen cells from SCID mice is dependent upon Ia⁺ cells. Unfractionated spleen cells (10^5 /well) or spleen cells that were depleted of Ia⁺ cells were cultured for 24 hr on anti-IFN- γ -coated plates in the presence of bDNA ($10 \mu\text{g/ml}$). IFN- γ -secreting cells were determined by Elispot.

sponse seen in response to stimulation with IL-12. In the absence of bDNA, macrophages (whether activated with IFN- γ or not), induced no NK cell IFN- γ secretion above background (data not shown). Although activated macrophages or IL-12 increased the IFN- γ response frequency, neither of these treatments appreciably altered Elispot size or intensity.

The IFN- γ response to bDNA is IL-12 dependent and IL-10 sensitive. The results presented in Figs. 3 and

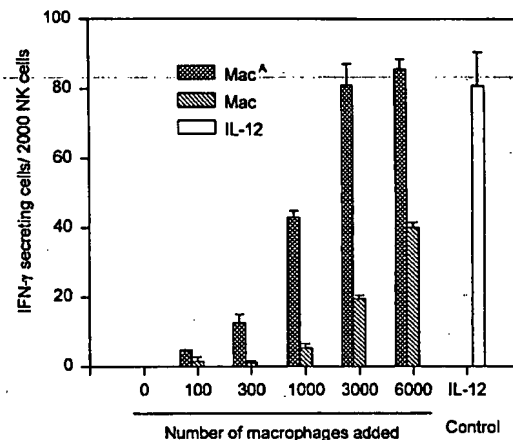


FIG. 5. The effect of macrophage concentration on the NK cell IFN- γ response to bDNA. Purified NK1.1⁺ cells ($2000/\text{well}$) were cocultured with varying numbers of macrophages. Adherent cells were isolated by plate adherence and, in some cases, preactivated by a 1-hr pulse with 100 u IFN- γ . Following removal from the plate, adherent cells were washed five times and macrophages were identified on the basis of bright staining with Mac 1. Cultures of NK cells and FACS-purified macrophages were stimulated with bDNA ($10 \mu\text{g/ml}$) or IL-12 (1 ng/ml) IFN- γ -secreting cells were determined by Elispot.

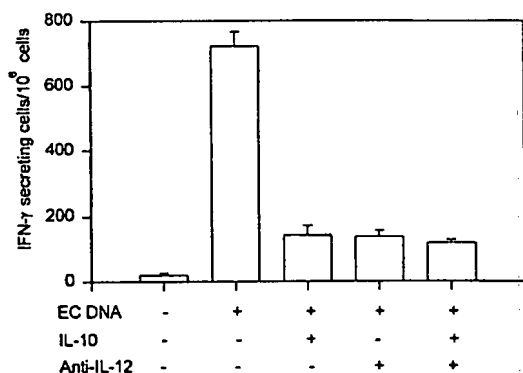


FIG. 6. b-DNA-induced IFN- γ production is IL-12 mediated and IL-10 sensitive. Elispot wells containing 10^5 splenocytes were stimulated with bDNA ($3 \mu\text{g/ml}$) in the presence or absence of anti-IL-12 ($10 \mu\text{g}$ each of 4 anti-IL-12 mAbs) or recombinant murine IL-10 (1 ng/ml). IFN- γ -secreting cells were identified after 24 hr. The results represent the mean \pm SEM of triplicate cultures.

5 suggested that bDNA stimulated macrophages to produce a soluble factor which, in turn, induced NK cell IFN- γ production. When anti-IL-12 or recombinant IL-10 was included in the spleen cell cultures that were stimulated with bDNA, the bDNA-induced IFN- γ response was reduced by over 80% (Fig. 6). These findings suggest that IL-12 is a critical intermediate in the NK cell response to bDNA, and that IL-10 may serve as a physiologic inhibitor of bDNA-induced cytokine production.

To define further the role of macrophages/accessory cells, we examined bDNA-induced IL-12 production by unfractionated spleen cells, purified macrophages, and by spleen cells previously depleted of macrophages. The results presented in Fig. 7 show that macrophages are the major contributors to bDNA-induced IL-12 secretion within the splenocyte population. Neither CT DNA nor media alone (data not shown) induced measurable IL-12 secretion at the cell concentrations examined. To determine if bDNA-driven IL-12 secretion is associated with an increase in IL-12 mRNA, we used RT-PCR to identify IL-12 p40 mRNA in bDNA-stimulated macrophages. Figure 8 shows that bDNA induces IL-12 mRNA in splenocytes, while CT DNA does not. The IL-12 response to bDNA was abolished by depletion of Mac 1^+ cells (by cell sorting). Additionally, FACS purified Mac 1^+ cells produced IL-12 mRNA following stimulation with bDNA. Thus, splenic macrophages are the major IL-12 producers following bDNA challenge, and bDNA-induced IL-12 production is associated with an increase in IL-12 p40 mRNA.

IL-12-stimulated NK cells show increased IFN- γ production when exposed to bDNA. Although NK cells alone do not produce IFN- γ in response to bDNA (Fig.

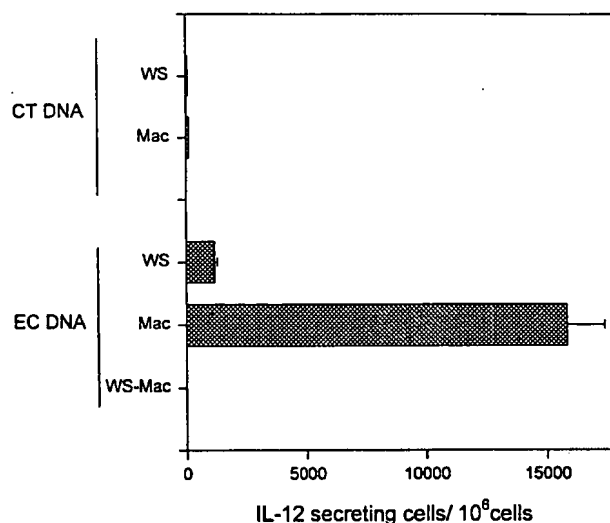


FIG. 7. Frequency of IL-12-secreting cells in unfractionated splenocytes (WS), macrophages, and splenocytes depleted of macrophages (WS-Mac) (see Materials and Methods). Cultures of 10^3 – 10^4 cells were established in Elispot wells (coated with anti-IL-12), and cultures were stimulated overnight with CT DNA or bDNA ($10 \mu\text{g/ml}$). Elispots were developed as detailed. To extrapolate the frequency of IL-12-secreting cells, results from wells with 15–50 spots were used in calculations.

5), we found that splenocytes stimulated with both IL-12 and bDNA secreted more IFN- γ than similar cultures stimulated with IL-12 alone. In contrast, CT DNA did not augment IL-12-induced IFN- γ secretion (data not shown). To explore the possibility that macrophage-derived IL-12 rendered NK cells competent to respond to bDNA, we examined the response of purified NK cells to varied concentrations of IL-12 in the presence or absence of bDNA. The results presented in Fig. 9 show that NK cell stimulation with bDNA in cultures

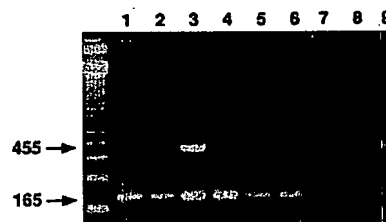


FIG. 8. Exposure to bDNA increases IL-12 mRNA levels in splenic macrophages. Splenocytes that were not sorted but were run through the cell sorter (10^5 in 0.2 ml) were cultured with media (lane 1), $3 \mu\text{g/ml}$ CT DNA (lane 2), or $3 \mu\text{g/ml}$ bDNA (lane 3). Sorted splenocytes depleted of Mac $^+$ cells (10^5 in 0.2 ml) were cultured with medium (lane 4), CT DNA (lane 5), or bDNA (lane 6). Purified Mac $^+$ cells (10^4 in 0.2 ml) were stimulated with medium (lane 7), CT DNA (lane 8), or bDNA (lane 9). After 2 hr, total RNA was reverse transcribed and cDNA was subjected to PCR amplification using primers for IL-12 and HGPRT (see Materials and Methods).

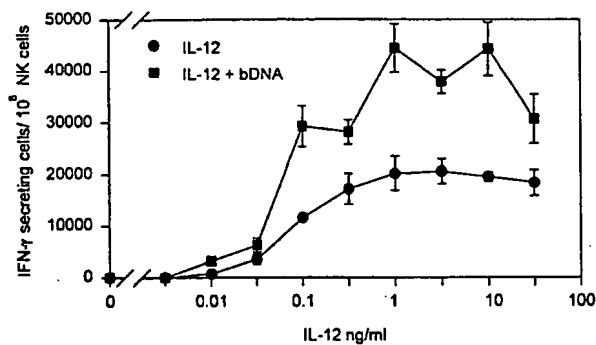


FIG. 9. IFN- γ secretion by NK cells in response to IL-12 or IL-12 + bDNA. NK cells were purified by flow cytometry, and 2000 cells were cultured for 24 hr on anti-IFN- γ -coated plates with IL-12 at the concentrations indicated in the presence or absence of bDNA (10 μ g/ml). The number of IFN- γ -secreting cells was determined by Elispot assay. This is one of four similar experiments.

containing as little as 0.1 u/ml of IL-12 promotes an IFN- γ response that exceeds the maximum response seen with much higher concentrations of IL-12. This finding indicates that IL-12 renders NK cells responsive to bDNA; this would allow bDNA to induce an IFN- γ response both indirectly via IL-12 stimulation and directly by costimulation of IL-12-activated NK cells.

DISCUSSION

The production of IFN- γ plays a critical role in determining the outcome of inflammatory responses. In the case of certain infectious diseases, an inadequate IFN- γ response or inability to respond to IFN- γ is associated with persistent infection or increased host mortality (2–8). *In vivo* manipulations that increase IFN- γ production have, in some cases, resulted in increased host resistance to pathogens (31–33) although T_H1 -mediated actions independent of IFN- γ may also contribute to the global state of host resistance (34). In addition to promoting host resistance, IFN- γ also plays an initiating role in the sepsis syndrome. Treatment regimens that increase IFN- γ production increase the sensitivity of mice to endotoxin shock (10, 35), while strategies designed to decrease IFN- γ production (such as IL-10 treatment) protect mice from shock (36). Mice with disrupted IFN- γ receptors are resistant to endotoxin shock (9), while mice with disrupted IL-10 genes (and excessive induced IFN- γ production) are hypersensitive to LPS-induced shock (37). These findings suggest the amount of IFN- γ produced in response to microbial challenge may determine whether the outcome is host protection or inflammation-induced morbidity.

In our previous study, we showed that bDNA induced NK cell IFN- γ production *in vivo* and increased the

susceptibility of mice to endotoxin shock (21). The experiments presented here show that macrophage-derived IL-12 plays a major role in promoting/augmenting the bDNA-induced IFN- γ response. Our findings are consistent with a recent study showing that IL-12 and TNF- α induced by bDNA promoted IFN- γ and IL-12 production by splenocytes (38). Here, we have used flow cytometry to establish that splenic macrophages are the major source of bDNA-induced IL-12, and we show that bDNA-induced IL-12 is responsible for driving the NK cell IFN- γ response.

Both our previous *in vivo* experiments and the present *in vitro* experiments examine the response to microgram quantities of free, nuclease-sensitive bacterial DNA. Although free DNA is cleared very rapidly from the circulation (39, 40), a significant proportion of free DNA or oligonucleotides is cleared by the reticuloendothelial system (40, 41) and could initiate an IL-12-driven NK cell IFN- γ response. Additionally, DNA contained within prokaryotic organisms is engulfed and partially degraded by phagocytic cells. Of note, polymorphonuclear leukocytes engulf and kill bacteria but do not completely degrade the bDNA (42). In this situation, bDNA fragments from killed intracellular organisms could be released into the cytoplasm of phagocytic cells. In this study, we analyzed individual IFN- γ -secreting cells rather than the total amount of cytokine secreted. In order to measure IFN- γ secretion by FACS-purified NK cells (which compose less than 2% of the splenocyte pool), we studied individual wells that contained 2000 NK cells. The amount of IFN- γ produced by this number of purified NK cells was below the amount that could be reliably measured by ELISA. An additional problem with ELISA analysis of supernatants is that cells present in the culture may express cytokine receptors which may artificially lower the estimate of the total cytokine present. The Elispot does not quantitate total cytokine, but does accurately measure the response in terms of cytokine-producing cells. Our maximum IFN- γ response frequency of purified NK cells was never more than 5%. This may be a consequence of mechanical sorting of the cells or may be due to the possibility that a subset of NK cells is responsive to IL-12.

Although the mechanism by which bDNA induces IL-12 is unknown, our findings that bDNA increases the level of IL-12 p40 mRNA suggests that bDNA may induce IL-12 gene transcription. It is possible that DNA fragments could interact with intracellular molecules that regulate the transcription of genes such as IL-12. The observed effect of bDNA on IL-12 mRNA expression could be mediated by either increased transcription or stabilization of the IL-12 message. Studies by others show that bDNA ingested by macrophages results in the appearance of NF- κ B in the nucleus (43).

Since NF- κ B has been implicated in IL-12 transcription (44), it is possible that bDNA directly interacts with transcription factors.

Our studies also show that preactivation of macrophages (with a pulse of IFN- γ) results in a marked increase in NK cell IFN- γ production when NK cells and macrophages are treated with bDNA. The augmentation of NK IFN- γ production is presumably due to increased IL-12 production since anti-IL-12 blocks this effect. This finding is consistent with the work of others which shows that IFN- γ promotes macrophage IL-12 production in response to mycobacteria (45) and that IFN- γ treatment results in activation of the monocyte IL-12 p40 promoter (46). Since IL-12 augments NK cell IFN- γ production, and since IFN- γ can increase macrophage IL-12 production, bDNA-induced production of IFN- γ may initiate and participate in a positive feedback loop. This could result in increasing amounts of IFN- γ as long as bDNA is available to stimulate macrophages or costimulate NK cells. In addition to confirming the importance of IL-12 in the NK cell IFN- γ response, our study also shows that NK cells that are exposed to IL-12 show a higher frequency of IFN- γ -secreting cells if bDNA is also present in the culture. This finding suggests that IL-12 renders NK cells responsive to bDNA, allowing IFN- γ nonproducers to become IFN- γ producers when both bDNA and IL-12 are present. The mechanism responsible for this augmentation is unclear.

In addition to possibly influencing the response to infectious agents, bDNA may also influence the expression of autoimmune inflammatory states. Our earlier studies showed that bDNA can function as a polyclonal B cell activator (17, 47). Studies by others have established that IL-12 is associated with inflammatory responses in experimental diabetes (48), multiple sclerosis (49), and experimental colitis (50). Since bDNA is a potent inducer of IL-12, it is possible that oligonucleotides derived from prokaryotic DNA could initiate and/or intensify some autoimmune inflammatory responses.

The ability of bDNA (or specific oligonucleotides) to induce IL-12 and IFN- γ production also has potential utility in the arena of vaccine development. Since both of these cytokines are important in directing the T cell compartment toward a more T_H1 response (51–54), nuclease-resistant nucleic acids could be included in vaccines where a strong cell-mediated immune response is desired or in immunotherapy designed to minimize the IgE response (as in allergic conditions). Thus, bDNA or derived oligonucleotides may serve as important modulators of inflammatory responses. Future studies will focus on ways to minimize unwanted effects (as in sepsis) and on ways to harness the potentially useful adjuvant effects of bDNA.

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